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(54) Title: HETEROBIFUNCTIONAL REAGENTS AND CONJUGATES WITH OXAALKYLENE UNITS FOR AMPHIPHILIC BRIDGE STRUCTURES

(57) Abstract

A conjugate substance (A-B-A'), where A and A' are residues from organic compounds F and F'; at least one of which being a polymer (carrier) and said compounds having properties that are retained in the conjugate and -B- being a bridge that covalently binds A to A'. The bridge -B- comprises the structure $-S_rRCONHCH_2CH_2(OCH_2CH_2)_nO(CH_2)_mCOY-$ (I); (i) n is often an integer 1-20 that is uniform for bridges linking identically located positions in individual molecules of the substance; (ii) m = 1 or 2; (iii) R = an alkylene group (1-4 carbon atoms) that possibly is substituted with one or more (1-3) hydroxy(OH) groups; (iv) = sulfur in the form of a thioether (r = 1) or disulfide (r = 2), and S_r binding to saturated carbon atoms in both directions; (v) Y is -NH-, -NHNH- or -NHN=CH- that in their left ends bind to CO and in their right ends to saturated carbon atom or to a carbonyl group (only -NHNH-). A bifunctional coupling reagent complying with the formula $Z_1RCONHCH_2CH_2(OCH_2CH_2)_nO(CH_2)_mZ'_1$ (III); (i) n is an integer, often 1-20; (ii) m is an integer 1 or 2, preferably 1; (iii) Z_1 = a SH-reactive electrophile or thiol (SH-) or protected thiol; (iv) R = an alkylene group (1-4 carbon atoms, that possibly is substituted with one or more (1-3) hydroxy(OH) groups; and (v) Z'_1 is activated carboxy. A polyether complying with the formula $XCH_2CH_2(OCH_2CH_2-)_nOCH_2Y$ (IV) where n is an integer 2-20, preferably 3-10. X is H_2N- or substituted H_2N- that is transformable to H_2N-, preferably by hydrolysis or reduction. Y is carboxy or a group that is transformable to carboxy.

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HETEROBIFUNCTIONAL REAGENTS AND CONJUGATES WITH OXAALKYLENE UNITS FOR AMPHIPHILIC BRIDGE STRUCTURES

The term conjugate is a c mm n designation f r substances prepared by covalently linking different or identical organic compounds together so that properties from the compounds will be conferred to the conjugate.

The conjugate substance, the reagent and the novel polyethers of the present invention have as the common structural feature sequentially linked ethoxy groups, i.e. the structure $-(OCH_2CH_2)_n-$, where n is an integer = or > 1.

The conjugate of the invention complies with the general formula A-B-A' wherein A and A' comprise residues from organic compounds F and F', respectively. At least one of the compounds is a polymer, the polymeric structure of which being present also in the residue. B represents an organic bridge linking A and A' together and comprising the structure $-(OCH_2CH_2)_n-$. A and A' may comprise several identical residues originating from the compounds to be conjugated (F and F').

For the preparation of conjugates one often utilizes hetero- or homobifunctional reagents of the type Z-B'-Z', where Z and Z' are reactive functional groups that are inert with respect to reaction with each other (in pairs they are nucleophilic or electrophilic) and B' is an inert bridge. By the term inert is contemplated that the bridge is stabile and has no groups that are able to neutralize the reactivity of Z and/or Z'.

In order to prepare conjugates in which one of the compounds (F or F') is a polymer, it is difficult to prepare uniform conjugate substances. The substances obtained will often consist of a mixture of more or less similar conjugate molecules. Common varieties of the individual molecules of a given conjugate substance are: different numbers of A bound to one and the same A' and vice versa, different binding positions, B exists as inter- and/or intramolecular cross-linkings tc.

Th length and th structure of the bridge -B- ar of great importanc in order to confer pr perti s of th compounds to the conjugate. For compounds that are poorly soluble in water, a hydrophobic bridge structure may result in conjugates that are insoluble and/or poorly active. If the bridge is too short and biological active compounds are conjugated, the activity will often be impaired. In the extreme case an erroneous or too short bridge may completely deteriorate the activity.

The structure $-(OCH_2CH_2-)_n$ is present in polyethylene glycol (PEG). The molecular weights of PEGs are given as a mean values, i.e. PEG is normally a mixture of various molecules in which the integer n varies. PEGs have appeared to have unique amphiphilic properties which can be utilized when they are linked to biopolymers.

The structure $-(OCH_2CH_2)_n-$ is also present in certain known amino-PEG-carboxylic acids. For instance $NH_2CH_2CH_2(OCH_2CH_2)_nO(CH_2)_2COOH$ with n = 1-10 (Houghton and Southby, Synth. Commun. 19(18)(1989)3199-3209) that have been suggested as starting material for the preparation of cyclic polyether amides. The authors have also suggested that homologues with m > 2 may find the same application. $NH_2CH_2CH_2(OCH_2CH_2)_2OCH_2COOH$ has been suggested as starting material for macrocycle-based ethers (Jullien et al, Tetrahedron Letters 29(1988)3803-06.

Recently (20.1.91) amino-PEG-carboxylic acids of the formula $NH_2CH_2CH_2(OCH_2CH_2)_nOCH_2COOH$ (n = 1-10), some of their reactive derivatives, and protein conjugates prepared from them have been disclosed (EP-A-410,280).

Conjugates exhibiting the structure $-(OCH_2CH_2)_n-$ in the bridge have been synthesized earlier than our priority date. Slama and Rando have linked cholesterol to a monosaccharide through both hydrophilic and hydrophobic bridges (Biochemistry 19(1980)4595-4600 and Carbohydrate Research 88(1981)213-221; a h t robifuncti nal c upling reagent). For th ir purp ses the best conjugates had the bridge:

$$(-NHCH_2CH_2(OCH_2CH_2)_nOCH_2CO-)_{n'},$$

where n = 1 and n' = 1 or 2. Slama and Rand did not manifold the $-OCH_2CH_2-$ structure by increasing the integer n. For unknown reason they instead duplicated the complete structure $-NHCH_2CH_2(OCH_2CH_2)_nOCH_2CO-$ by making n' equal 2. Enzyme-antibody conjugates containing the bridge $-(OCH_2CH_2)_n-$ in which n may be various integers are known (EP-A-254,172 and FR-A-2,626,373). In the latter application commercially available polyethylene glycol (PEG) was used as one of the starting materials for the coupling reagent. This has rendered it difficult to obtain uniform conjugate substances with respect to the length of the bridge. Oxaalkylene structures, e.g. $-OCH_2CH_2-$, have been suggested in heterobifunctional reagents for the selective coupling at aldehyde groups and thiol groups, respectively (EP-A-240,200 and WO-A-89/12624).

In addition to the publications given above the Swedish Patent Office has cited in an International Type Search Report: EP-A2-345,789, WO-A1-88/03412, Biochem. Biophys. Res. Comm. 164(1989-11):3, as publications of particular relevance with regard to the claims of the priority applications.

The present invention provides conjugate substances in which the spectrum of individual molecules have an improved uniformity and a good bioavailability, particularly in hydrophilic aqueous media. This goal is achieved by inserting an amphiphilic bridge structure of uniform and defined length. The conjugates of the invention is particularly adapted for **in vivo** and **in vitro** diagnostic uses as well as for therapeutic uses (drugs).

The conjugate of the invention is characterized in that the bridge -B- comprises the structure

$$-S_rRCONHCH_2CH_2(OCH_2CH_2)_nO(CH_2)_mCOY- \qquad (I)$$

Th free valencies in f rmula (I) link to A and A', resp ctively. This takes plac either directly or thr ugh furth r dival nt inert structures that are c mprised within the bridge -B-. The length of -B- is usually sh rter than

180 at ms, such as < 100 atoms, but l nger than 13, preferably longer than 16 atoms.

n is an integer > 0, e.g. 1-20, and such that n is uniform for bridges linking identical positions together in individual molecules of the conjugate (conjugate substance). m is 1 or 2. From the synthetic point of view n is preferably 10 or < 10. In order for the conjugate to express the unique amphiphilic properties of PEG, the integer n should be higher than 2, preferably higher than 3 or higher than 4. Thus based on different combinations of criteria the intervals for the integer n may be 1-20, 1-10, 1-9, 2-20, 2-10, 2-9, 3-20, 3-10, 3-9, 4-20, 4-10, 4-9, 5-20, 5-10, and 5-9.

S_r binds directly to a saturated carbon atom at each of its valencies. r = 1 or 2, that is S_r represents a disulfide group or a thioether group. If S_r is binding directly to A, one of the sulphur atoms may originate from F.

Y is -NH-, -NHNH- or -NHN=CH- that at their left ends bind to the CO group shown in the right terminal in formula I and at their right ends to a saturated carbon atom or to a carbonyl group (only when Y equals -NHNH-). The atoms binding to the right end of Y are not shown in formula I.

R is preferably alkylene (having 1-4 carbon atoms, often 1 or 2 carbon atoms), that possibly is substituted with one or more (1-3, in the preferred case < 2) hydroxy (OH) groups. At most one oxygen atom is bound to one and the same carbon atom in R. With respect to availability in hydrophilic media R may in many cases be equivalently substituted for a higher alkylene selected from the group comprising straight, branched and cyclic alkylene, with th provision that the higher alkylene shall exhibit hydrophilic substituents compensating for an enhanced hydrophobicity.

A further condition is that for r = 1, B may c ntain a 1-aza-2,5-dioxo-cyclopentan-1,3-diyl group that at its 3-position binds to the sulphur atom and at its 1-position to

R, or the analogous 1,4-diyl group that at its 4-position binds to the sulphur atom.

According to a preferred embodiment the bridge -B- must not contain any aromatic ring.

The polymer may be a synthetic one or a biopolymer. The term polymer stands for a compound in which three or more, preferably more than 10, repeating monomeric units bind sequentially to each other. The term polymer also encompasses inorganic polymers, such as glass and other polymeric silicates.

Examples of synthetic polymers are poly(hydroxyalkyl (meth)acrylate), poly((meth)acrylamide), poly(vinyl alcohol), etc and derivatized forms of these polymers.

The term biopolymer means a polymer in which the basic skeleton is of biological origin. The expression encompasses also biopolymers that have been derivatized. Biopolymers exhibit as a rule nucleic acid or polysaccharide and/or polypeptide structure. Proteins including polypeptides (e.g. albumin and immunoglobulins) and polysaccharides, e.g. soluble and insoluble ones (dextran, starch, heparin cellulose etc), are important.

Polymers of immediate interest (e.g. those of polypeptide and/or polysaccharide structure) usually have functional groups such as hydroxy (-OH), carboxy (-COOH), amino (primary or secondary) and/or mercapto (-SH). To the extent that a given polymer does not have the appropriate functional group, chemic l modification can as a rule be carried out to the effect that the group will be inserted.

The compounds F and F′ are selected according to the properties that they shall confer to the conjugate. The compounds may thus be: carrier compounds, bioaffinity compounds, analytically detectable compounds, compounds that are insoluble or soluble in aqueous media, th rapeutically active compounds (drugs), enzymes, immune stimulators, toxins etc. Particularly imp rtant toxins are the peptide cytotoxins that ex rt their ffect intracellularly and thus comprise one peptide segment

r sponsible f r penetration of the cell wall and another segm nt for the intracellularly toxicity (Diphteria toxin, ricin, Pseudomonas exotoxin etc). Another type of peptide toxins are those that exert their effect through immune stimulation (e.g. by activating cytotoxic T-cells via simultaneous binding to T-cells and cells carrying Class II MHC antigens). An example of the latter type is staphylococcal enterotoxin A.

Bioaffinity compounds, i.e. compounds that exert biospecific affinity, are particularly interesting, because they as a part of a conjugate may be utilized as a targeter for their bioaffinity counterparts. Examples are antibodies and their antibody active fragments and corresponding antigens/haptens, Fc-fragments/Fc-parts of immunoglobulins (Ig) and corresponding receptors (for instance IgG binds to Protein A and G), avidin/strepavidin and biotin, lectins and corresponding carbohydrate structures, enzymes and respective substrate, coenzyme, cofactor, and cosubstrate, etc. Antibodies of various classes (in particular IgG) and subclasses and various specificities may be one part of the conjugate according to the invention. The specificities of the antibodies (and fragment thereof) may e.g. be for tumour cells and/or tumour antigens/haptens, hormones, hormone receptors etc.

Particularly interesting insoluble polymers are those ones that are used as adsorbents in connection with chromatography, immunoassays, blood fractionation etc.

Within the field of **in vitro** and **in vivo** diagnostics, conjugates between an analytically detectable compound and a compound (targeter) showing biospecific affinity are of great importance for detecting and localizing the counterpart to the targeter. The analytically detectable compounds may be radioactive, enzymatically active (including enzyme substrates, cosubstrat s, c enzymes etc), fluorogenic, chemiluminogenic, bi chemiluminogenic, particulate (e.g. latex) tc.

For therapeutic purposes bioaffinity c mpounds may be conjugated to drugs and other substances that exert a therapeutic effect.

For the synthesis of the conjugate of the invention we have developed a novel heterobifunctional reagent of the type Z-B'-Z'. This reagent complies with the general formula (II):

$$Z_1RCONHCH_2CH_2(OCH_2CH_2)_nO(CH_2)_mZ_1' \qquad (II)$$

m and n have the same meaning as above for formula (I). Preferably m and n are uniform, i.e. the reagent substance is not a mixture of compounds having different m and different n. R is an alkylene group of the same meaning as above. Z_1 is an HS-reactive electrophilic group, thiol (-SH) or protected thiol (e.g. AcS-), with the provision that a thiol group and a hydroxy group must not be bound to one and the same carbon atom in R. Examples of HS-reactive electrophilic groups are:

(i) halogen that is bound to a saturated carbon atom, preferably in the form of an alfa-halo-alkylcarbonyl (e.g. Z_1CH_2CO-, where Z_1 preferably is bromo or iodo);

(ii) activated thiol, preferably a so called reactive disulfide (-SSR_1) that is bound to a saturated carbon atom;

(iii) 3,5-dioxo-1-aza-cyclopent-3-en-1-yl. With respect to reactivity against thiol groups one can equivalently use other carbon-carbon double bonds that form conjugated pi-electron systems with a carbonyl group, a nitro group or a cyano group instead of 3,5-dioxo-1-aza-cyclopent-3-en-1-yl;

Reactive disulfides are well known in the context of synthetic chemistry (See EP-A-063,109, 064,040, and 128,885). R_1 is defined by the chemical reaction between -S-S-R_1 and HS- releasing R_1-SH that is thermodynamically stabilized t be withdrawn from further thiol-disulfide exchange r actions. Many thiol c mp unds (R_1'SH) comply with this crit ri n by spontaneously tautomerizing to a thione form in aqueous solutions, i.e. the thi ne form is

more stabile than the corresponding thi l form. One prer quisite for this type of tautomerization may be that the sulfur atom of the thiol group is bound to a carbon atom that constitutes a part of an aromatic ring that (a) is heterocyclic having the thiol sulfur atom located at a distance of an odd number of atoms from a heteroatom in the ring, or (b) is non-heterocyclic and substituted with electron-withdrawing groups.

Examples of R_1 are 5-nitro-2-pyridyl, 5-carboxy-2-pyridyl, 2-pyridyl, 4-pyridyl, 2-benzthiazolyl, 4-nitro-3-carboxyphenyl and the N-oxides of the pyridyl groups just mentioned.

Z_1' is activated carboxy, i.e. an electrophilic group. Examples are carboxylic acid halides (-COCl, -COBr, and -COI), mixed carboxylic acid anhydrides ($-COOOCR_1$), reactive esters, such as N-succinimidyloxycarbonyl, $-C(=NH)-OR_2$, 4-nitrophenylcarboxylate ($-CO-OC_6H_4NO_2$) etc. R_1 and R_2 may be lower alkyl (C_1-C_6) and R_2 also benzyl or C_2-C_3 alkylene with one of its valencies substituting H in NH (giving cyclic structures such as in oxazolin-2-yl that possibly may be substituted with lower alkyl (C_1-C_6) or benzyl in its 3- and/or 4-position).

The Z_1' terminal may be reacted selectively with a compound F′ showing a nucleophilic group selected among:

(i) $-NHR_1$, such as in primary and secondary amines (R_1 is selected from hydrogen and lower alkyl (C_1-C_6)) and in hydrazine/hydrazide, i.e. NH_2NH_2 and compounds in which NH_2NH- and NH_2NH-CO- are bound to an aliphatic carbon atom, preferably saturated.

(ii) -OH, such as in an alcohol.

The chemical reaction at the Z_1' terminal of the reagent (II) with a compound F′ means that F′ will become covalently attached to $-(CH_2)_m$ in formula (II) via an amide group or a hydrazide group (-CONHNH- and -CONHN=C-, resp ctively) for groups according to (i) ab ve and via an ester group for groups according to (ii) ab ve. When appropriat , Z_1 may then b transformed (reduced) to a

thiol group in a thi l-disulfide exchang reaction. In the latter case F' will become thiolated.

The Z_1 terminal may selectively be reacted with a compound F having a thiol group (SH) or a HS-reactive electrophilic group. If F has a thiol group reaction can take place directly. In the final product compound F will be bound to R in formula (II) via a thioether (-S-) or disulfide (-S-S-) group.

The use of the reagent (II) is carried out in a manner known per se. The reaction medium is selected so that side reactions of Z_1 and Z_1' are avoided. When F and/or F' are biopolymers it is preferred to run the reaction in aqueous media, and in order to achieve selectivity pH shall be 8-9.5 for reaction at Z_1' and < 8 for reaction at Z_1. Aprotic media are often inert against Z_1' which means that they generally speaking are the preferred ones. The result will be a conjugate in which F and F' are linked together through a bridge complying with formula I.

Extended bridges can be introduced by reacting the reagent of formula (II) at either its Z_1 or Z_1' terminal by suitable bifunctional compounds. For instance, if Z_1' is reacted with an alkylene diamine, alkylene dihydrazine, alkylene dihydrazide etc and only one of their H_2N- groups is consumed, the remaining free NH_2-group can be used for reaction with other compounds, e.g. F or F' (exhibiting karboxy, optionally after activation).

Elongation of the bridge may also be accomplished by reacting the compounds F and/or F' with appropriate bifunctional reagents of the type A-B'-Z' (see page 1) prior to linking them together by the use of the reagent of the invention. B' may be selected from the same group as R above. If each of the compounds F and F' are initially reacted with the same reagent Z-B'-Z' at the Z' group and then linked together thr ugh the s introduced Z group the group B' will appear twice in the c njugate (head to head linking).

Known techniques encompass a large number of bifunctional reagents that are useful for chain elongation, either starting from a reagent of formula (II) or from one of the compounds F and F′. Specific examples are N-succinimidyl 4-(N-malein-imidyl)-butyrate, N-succinimidyl iodoodacetate, N-succinimidyl S-acetyl-2-mercaptoacetate, and N-succinimidyl 3-(pyridyl-2-dithio)propionate, alkylene diamine, alkylene diacylhydrazide etc. Alkylene has the same meaning as previously.

In a manner known per se for the synthesis of conjugates, a vicinal diol, e.g in a carbohydrate structure, may be oxidized to two aldehyde groups and subsequently reacted with a $-CONHNH_2$ group to give a -CONHN=C- group. Important compounds F and F′ having carbohydrate structures are the glycoproteins. The group $-CONHNH_2$ may be present in a bifunctional reagent Z-B′-Z′, optionally after reaction with a polymer.

Chain elongation by starting from a reagent of the present invention may result in conjugates in which -B- is:

(1) $-\underline{C}OR'-S_r-RCONHCH_2CH_2(OCH_2CH_2)_nO(CH_2)_mCOY-$;
$\underline{C}O$ binds to NH,

(2) $-COR'-S_r-RCONHCH_2CH_2(OCH_2CH_2)_nO(CH_2)_mCONHNR''NH\underline{N=}$;
$\underline{N=}$ is usually bound to a sp^2-hybridized carbon atom in A which means that the structure -N= is part of the structure -C=N-. This structure may have been formed by reaction between an aldehyde group and a NH_2-NHCO- group,

(3) $-\underline{C}O(CH_2)_mO(CH_2CH_2O)_nCH_2CH_2NHCOR'-S_r-$(cont.)
$-RCONHCH_2CH_2(OCH_2CH_2)_nO(CH_2)_mCOY-$;
$\underline{C}O$- is bound NH,

(4) $-CO(CH_2)_mO(CH_2CH_2O)_nCH_2CH_2NHCOR'-S_r-$(cont.)
$-RCONHCH_2CH_2(OCH_2CH_2)_nO(CH_2)_mCONHNR''NH\underline{N=}$;
$\underline{N=}$ is bound as above in (2),

M re variations are possible. r has th same meaning as above.

By varying the reagents that are empl yed, different chain elongations may be constructed starting fr m the Y-terminal.

R and R' are alkylene selected in the same way as R in formula (I).

The reagent (Formula II) can be prepared starting from compounds complying with formula III:

$$NH_2CH_2CH_2(OCH_2CH_2)_nO(CH_2)_mCOOH \qquad (III)$$

m equals an integer 1 or 2. n equals an integer 1-20, such as 2-20.

The synthesis of certain compounds complying with formula III with m = 1 and 2, and n = 1-10 have been described before (Jullien et al, Tetrahedron Letters 29(1988)3803-06; Houghton and Southby, Synth.Commun. 19(18)(1989)3199-3209; EP-A-410,280 (publ. 20.1.91); and Slama and Rando, Biochemistry 19(1980)4595-4600 and Carbohydrate Research 88(1981)213-221).

The reagents complying with formula II can be synthesized by reacting a compound of formula (III) with a bifunctional reagent of formula Z-B'-Z' and known per se (see page 1), where $Z = Z_1$, B' = R that is as previously defined, and Z' = activated carboxy. See above for suitable reagents. After the reaction the -COOH function is transformed to an activated carboxy group, e.g. Z_1' = activated ester, such as N-succinimidyloxycarbonyl, 4-nitrophenyloxycarbonyl, 2,4-dinitrophenyloxycarbonyl etc.

Compounds complying with formula III (m = 1 and n = 2-20) and derivatives having the NH_2- and/or -COOH groups replaced with groups that easily can be transformed to NH_2- and -COOH groups, respectively, are novel and relate to a separate aspect of the present invention. This aspect provides a number of discrete bifunctional substances having pronounced amphiphilic properties, i.e. the property of being simultaneously soluble in wat r and organic solv nts and lipids. This is a d sirable property for bridge forming reagents that are to be used f r the preparation of conjugates involving biomolecules.

The novel compounds of formula (III) and their novel d rivatives comply with polyethers having the general formula:

$$XCH_2CH_2(OCH_2CH_2-)_nOCH_2Y \qquad (IV)$$

n is an integer 2-20, preferably 3-20. X is H_2N- including the protonated form thereof (^+H_3N-) or substituted H_2N- that is transformable to H_2N-, preferably by hydrolysis or reduction. Examples are unsubstituted amino (H_2N-); nitro; amido (= carbamido), such as lower acylamido (formylamido, acetylamido .... hexanoylamido) including acylamido groups that have electron-withdrawing substituents on the alpha carbon atom of the acyl moiety and then particularly CF_3CONH-, CH_3COCH_2CONH- etc; phtalimidyl which possibly is ring substituted; carbamato (particularly R_1'OCONH- and (R_1'OCO)(R_2'OCO)N-, such as N-(t-butyloxycarbonyl)amino (Boc), N-(benzyloxycarbonyl)amino and di(N-(benzyloxycarbonyl))amino (Z and diZ, respectively) which possibly are ring substituted; alkyl amino in which the carbon atom binding to the nitrogen atom is alpha to an aromatic system, such as N-monobenzylamino and dibenzylamino, N-tritylamino (triphenylmethylamino) etc including analogous groups where the methyl carbon atom (including benzylic carbon atom) atom is replaced with a silicon atom (Si), such as N,N-di(tert-butylsilyl)amino; and 4-oxo-1,3,5-triazin-1-yl including such ones that are substituted with lower alkyl in their 3- and/or 5-positions.

Above and henceforth R_1' and R_2' stand for lower alkyl, particularly secondary and tertiary alkyl groups, and a methyl group that is substituted with 1-3 phenyl groups that possibly are ring substituted. Lower alkyl and lower acyl groups have 1-6 carbon atoms.

Y is carboxy (-COOH including -COO$^-$) or a group that is transformable t carboxy, pr ferably by hydr lysis or oxidation. The most important groups are the ster groups in which the carbonyl carbon atom r the c rresponding atom in orto esters binds to the methylene group in the right

t rminal of formula (I). Examples are alkyl ester groups ($-COOR_1'$); orto ester groups ($-C(OR_3')_3$) and reactive est r groups, such as N-succinimidyloxycarbonyl, 4-nitrophenyloxycarbonyl, alkyl imidate groups ($-C(=NH)O-R_1'$) including 5-membered cyclic forms (oxazolin-2-yl) with or without lower alkyl in their 4- and/or 5-positions). R_3' has the meaning as previously given for R_1.

Other groups Y are -CHO, -CN, $-CONR_1'R_2'$, where R_1' and R_2' have the same meaning as previously, and $-CONH_2$.

The compound of the invention may be synthesized from known starting materials by combining methods that are known per se. Appropriate synthetic routes are:

A. Formation of the chain.

B. Transformation of terminal functional groups.

C. Transformation of a symmetric polyether to an unsymmetric ether.

D. Splitting of a bisymmetric chain into two identical fragments.

Convenient starting materials that have the repeating unit $-OCH_2CH_2-$ are commercially available. Examples are oligoethylene glycols having 2 to 6 repeating units. Other suitable compounds with identical terminal groups are corresponding dicarboxylic acids and diamines.

Convenient starting materials that have different terminal groups are omega-hydroxy monocarboxylic acids in which the terminal groups are spaced apart by a pure polyethyleneoxide bridge. Such compounds having up to 5 repeating units have been described in the prior art (Nakatsuji, Kawamura and Okahara, Synthesis (1981) p.42).

<u>A. Formation of the chain.</u>

Williamson's ether synthesis can be applied to the synthesis of chains having the repeating unit $-OCH_2CH_2-$ and identical or different terminal groups. The m thod means alkylati n of an alc hol (QZ'' + HOQ' --> Q-O-Q' or the other way round QOH + Z''Q' --> Q-O-Q'). By s l cting

(i) $Q = X'CH_2(OCH_2CH_2)_p$- in which X' is XCH_2- or a group that in on or mor steps is transformabl to XCH_2- and stabile under the conditions for Williamson's ether synthesis, and

(ii) $Q' = -(CH_2CH_2O)_rCH_2Y'$ in which Y' is Y or a group that in one or more steps is transformable to Y and stabile under the conditions applied.

p and r are 0 or positive integers such that $p + r = n$.

Williamson's ether synthesis may also be applied to the synthesis of the type of bisymmetric chains that is described in Part D.

For the case that Y' is not Y, one select Y' preferably among groups that are stabile against strong bases. For instance Y' may be $-CH_2OR_4'$, $-CH=CR_5'R_6'$, where R_4' are selected from lower alkyl groups (C_1-C_6), preferably secondary or tertiary alkyl groups of at most 5 carbon atoms and possibly substituted in their alpha positions with at most three phenyl groups that possibly are substituted, and R_5' and R_6' are hydrogen, lower alkyl (C_1-C_6) or phenyl that possibly is substituted.

In the formulas given above Z'' is a leaving group of moderate to high reactivity and may be halo, alkanesulfonate, arenesulfonate, preferably toluenesulfonate (tosylate), and perfluoroalkanesulfonate, preferably trifluoromethanesulfonate etc. Williamson's ether synthesis may be carried out in inert solvents and normally in the presence of a base - often strong bases, such as sodium hydride etc. By combining components of appropriate lengths one can in principle develop any chain $-(OCH_2CH_2-)_n$ by a sequence of elongation steps.

The chain can also be elongated with one OCH_2CH_2 unit through Michael addition of HOQ or HOQ' to a compound $X''-C(X''')=CH_2$, where Q and Q', respectively', have the sam m aning as previ usly. Th gr ups X'' and X''', respectively, have to be select d such that -OQ and OQ' are guided to the 2-carbon atom ($=CH_2$) in the compound $X''-C(X''')=CH_2$ and such that th terminal gr ups in Q and Q',

r sp ctively, are stabile during the conditi ns applied. X''' may be hydrogen and X'' may b a group that is easily transformable to amino or substituted amino, e.g. nitro. Alternatively X'' and X''', respectively, may be groups that after the transformation enable the group X''-C(X''')CH_2- to be converted to the group $HOOCCH_2$- or a derivative thereof. Preferably X'' is methylsulfinyl and X''' methylthio, and this requires hydrolysis of the product obtained in the addition step, said hydrolysis giving an aldehyde that subsequently may be oxidized to the corresponding carboxylic acid. The conditions for Michael addition are similar to those ones for Williamson's ether synthesis.

A third alternative for chain elongation is reduction of thione ester by the use of Raney Nickel or corresponding reagents having a high affinity for sulfur. Suitable thiono esters comply with the formula:

$$X'CH_2(OCH_2CH_2)_qOCH_2CS\text{-}(OCH_2CH_2)_s\text{-}CH_2Y'$$

or

$$X'CH_2(OCH_2CH_2)_pO\text{-}CSCH_2(OCH_2CH_2)_q\text{-}CH_2Y'$$

where X' and Y ' have the same meaning as previously and q and s are 0 or positive integers such that $q + s = n - 1$, where n has the same meaning as previously.

Thione esters can be synthesized through rearrangement of the corresponding thiol esters by the action of an alkylating agent, e.g. methyl iodide, dimethyl sulfate or diazo methane. Thiol esters may be synthesized by reaction of a carboxylic acid halide with a thiol compound. Another alternative is reaction of a carboxylic acid ester with a sulfur transferring reagent that is selective for double bonded oxygen when present in a carboxylic acid ester. Examples of such reagents are phosphorous pentasulfide or preferably Lawesson's reagent (2,4-bis(4-methoxyphenyl)-1,2,3,4-dithiaphosphetane-2,4-bis-sulfid). In this r arrangement the functional terminal gr ups must n t contain a carboxy oxygen.

B. Transformation of terminal groups.

A compound complying with the f rmula:

$$X'-CH_2-(OCH_2CH_2-)_nOCH_2Y'$$

where X'- is XCH_2- or a group that in one or more steps is transformable to XCH_2-, and -Y' is Y or a group that, in one or more steps is transformable to Y, may be subjected to reaction conditions that give the desired transformation and end product or where appropriate give an intermediary product that can be used in a coupling step.

Exemples of X' (besides XCH_2-) are hydroxymethyl, $R_4'OCH_2$-, $CR_5'R_6'$=CH-, where R_4', R_5' and R_6' have the same meaning as previously. In the case that X' is hydroxymethyl the transformation can take place, e.g. by reaction with a sulfonyl halogenide in the presence of a base and followed by reaction with ammonia and, if necessary, further reagents to give the appropriately substituted amino group, such as phtalimido. In the case that X'- is $R_4'OCH_2$- the group R_4' is removed, e.g. by acid catalyzed hydrogenolysis. The exposed hydroxymethyl group is then transformed to a group containing a nitrogen atom as previously given. In the case that X' is CR_5R_6=CH-, the =CH- of the alkenylene group is oxidized to an aldehyde group, e.g. by ozonisation. The aldehyde group is then transformed to an aminomethyl group by reductive amination.

Examples of -Y' (in addition to $-CH_2Y$) are hydroxymethyl, $-CH_2OR_4'$, $-CH=CR_5'R_6'$, where R_4', R_5' and R_6' have the same meaning as previously. In the case that Y' is hydroxymethyl the reaction may take place by oxidation to a carboxy group (-COOH). In the case that -Y' is $-CH_2OR_4'$, one removes R_4', for instance by acid catalyzed hydrolysis, and, if R_4' contains at least one alpha positioned phenyl, the removal can be performed by catalytic hydrogenolysis. The exposed hydroxymethyl group may then be transformed according to what has b n said above. Alt rnativ ly, on xidizes the group directly to the corresponding carboxy group (-COOH). In the cas that -Y' is $-CH=CR_5'R_6'$, the -CH= of the alk nylene group may be

oxidized to a carboxy group (-COOH), possibly via an intermediary aldehyde (e.g. by ozonisation) that may be further oxidized. The oxidations may be run in the presence of an appropriate tertiary alcohol, for instance t-butanol, in such a way that the corresponding ester will be formed directly.

C. Transformation of a symmetric polyether $X_1CH_2(OCH_2CH_2)_nOCH_2X_1$ to an unsymmetrical ether.

Symmetrical ethers can be synthesized from shorter polyethylenoxide ethers by chain elongation, for instance by the use of Williamson's ether synthesis. Transformation of one of the two identical groups X_1, where X_1 is X' or Y' as given previously, to another group can be performed by partial reaction of the desired kind and subsequent separation of the unsymmetrical product from the unreacted starting material and the double-reacted side product. Examples of such transformations are:

a) When X_1 is a carboxy group the dicarboxylic acid is transformed to the corresponding di(acyl halide) that in turn can be reacted with a deficient amount af ammonia followed by hydrolysis of the remaining acid halide groups. The formed amide carboxylic acid is separated from the reaction mixture whereafter its amide function is selectively reduced to an aminomethyl group. Alternatively, the separation is carried out after the reduction step by a sequence of ion exchange separation steps.

b) When X_1 is aminomethyl the diamino compound is reacted with a deficient amount of an acylating carboxylic acid derivative, such as carboxylic acid chloride or corresponding anhydride. The monoacylated product is then separated from the starting material and the diacylated product, whereafter its aminomethyl group is oxidized, for instance through th corresp nding ald hyde, to a carboxy group, and its acyl group removed by hydrolysis. A particularly useful variation of this meth d is th reaction with cyclic anhydride. In this latter case ion

exchange separation will become facilitated because the int rm diary product is an amino acid, the starting material a diamine, and the side product a dicarboxylic acid.

c) If X_1 is a group of moderate to high lipophilicity, for instance trityloxymethyl or allyloxymethyl, partial removal or transformation of one of the groups will facilitate separation by liquid partition due to a significant difference between the partition coefficients for the product, the starting material and the side product. Analogous critera are valid if the appropriately lipophilic group has been introduced onto to a symmetric polyether having hydrophilic groups X_1, for instance oligoethylene glycol that is monosubstituted with trityl.

d) When X_1 is hydroxymethyl the reaction can be performed by Williamson's ether synthesis, for instance by adding an excess of haloacetic acid. After the reaction the mixture is esterified with a suitable lower alcohol (C_1-C_6), for instance isopropanol. By selecting the proper alcohol, the differences between the partition coefficients (water and organic solvents) of the components in the reaction mixture will become more pronounced. This will facilitate the separation of the product from the starting material and from the disubstituted side product. For instance a diester will be transferred from an aqueous phase to an organic solvent in neutral solution, while the monoester will be transferred at an acid pH. For compounds that are relatively volatile, the major part of the starting material may be removed by destillation. Exemples are diethylene glycol or triethylene glycol.

D. Splitting of a bisymmetric chain into two identical <u>fragments.</u>

A compound having the formula:

A) $X'CH_2-(OCH_2CH_2)_n-OCH_2CH{=}CHCH_2O-(CH_2CH_2O)_n-CH_2X'$ or

B) $Y'CH_2-(OCH_2CH_2)_n-OCH_2CH{=}CHCH_2O-(CH_2CH_2O)_n-CH_2Y'$,

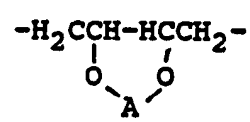
where X' and Y', respectively, have the same meaning as previously, provides a special case. The splitting is carried out by oxidation of the double bond; in case A directly to two identical carboxy groups and in case B to two identical aldehyde groups that in turn is transformed to aminomethyl groups by reductive amination.

The starting

$X'CH_2-(OCH_2CH_2)_n-OCH_2CH{=}CHCH_2O-(CH_2CH_2O)_n-CH_2X'$ and

$Y'CH_2-(OCH_2CH_2)_n-OCH_2CH{=}CHCH_2O-(CH_2CH_2O)_n-CH_2Y'$,

respectively, are preferably synthesized through Williamson's ether synthesis by reacting $HOCH_2CH{=}CHCH_2OH$ with two equivalents of $X'CH_2-(OCH_2CH_2)_n-Z''$ and $Y'CH_2-(OCH_2CH_2)_n-Z''$, respectively, where X', Y' Z'' and n have the same meaning as previously. An alternative route is to react $Z''-CH_2CH{=}CHCH_2-Z''$ with two equivalents of $X'CH_2-(OCH_2CH_2)_n-OH$ and $Y'CH_2-(OCH_2CH_2)_n-OH$, respectively. Analogous methods are also available. For instance one may employ compounds in which the group $-CH_2CH{=}CHCH_2-$ is replaced by the group



where A is a possibly substituted lower alkylidene (preferably C_1-C_6), preferably dimethylmethylene. In this case the group A is removed by acid hydrolysis, whereafter the intermediary 1,2-diol formed is oxidized, for instance by periodate and subsequent transformation of the aldehyde group formed to an aminomethyl group or to a carboxy group in a manner known per se.

The invention is defin d by the appending claims that are a part of the specification.

The exemplification below is divided into thre parts: Part 1 illustrates the synthesis of amino-PEG-carboxylic acids complying with formula IV (m = 1), Part II illustrates the synthesis of heterobifunctional reagents complying with formula II and of conjugates having a bridge structure according to formula I, and Part III sums up the results obtained for conjugates between the T-cell immune stimulator (superantigen) staphylococcal enterotoxin A (SEA) and antibodies directed against tumour antigens. Examples 5 and 6 (Part 2) illustrate the synthesis of a conjugate between an immunoglobulin and a peptide. Comparative experiments have shown that, by having a bridge according to the invention, the solubility of the conjugate will be increased and therefore also the availability of the peptide in aqueous media. The comparison has been made against a conjugate comprising the same peptide and antibody, but having a short hydrophobic bridge that is not according to the invention.

EXPERIMENTAL PORTION. PART 1.

Isopropyl 8-hydroxy-3,6-dioxa-octanoate (1).

Sodium (23 g, 1.0 mole) in form of chips was added in portions to diethylene glycol (500 ml) under nitrogen atmosphere. When the sodium had reacted completely, the mixture was cooled to room temperature and bromoacetic acid was added (76 g, 0.5 mole) under stirring. After 18 hours at 100°C the excess of diethylene glycol was distilled off at about 4 mm Hg. Thereafter isopropyl alcohol (400 ml) and in portions acetyl chloride (51 g, 0.65 mole) were added. After stirring for 18 hours at 65°C the mixture was cooled to room temperature and neutralized with sodium acetate (3.5 g, 0.15 mole). The mixture was filtered and the filtrate evaporated nearly to dryness, whereupon it was dissolved in water (200 ml). The water phase was extracted with 1,1,1-trichloro-ethane (3x50ml). The pooled organic phases were washed with water (20 ml). The product was extracted from the pooled water phases with dichloromethane (50 ml) that after evaporation gave an oil (55 g).

Isopropyl 11-hydroxy-3,6,9-trioxa-undecanoate (2).

Sodium (23 g, 1.0 mole) in form of chips was added in portions to triethylene glycol (700 ml) under nitrogen atmosphere. When the sodium had reacted completely, the mixture was cooled to room temperature and bromoacetic acid was added (76 g, 0.5 mole) under stirring. After 18 hours at 100°C the excess of diethylene glycol was distilled off at about 4 mm Hg. Thereafter isopropyl alcohol (400 ml) and in portions acetyl chloride (51 g, 0.65 mole) were added. After stirring for 18 hours at 65°C the mixture was cooled to room temperature and neutralized with sodium acetate (3.5 g, 0.15 mole). The mixture was filtered and the filtrate evaporat d nearly t dryness, whereupon it was dissolved in water (200 ml). The water phase was extracted with 1,1,1-trichloro-ethane (3x50ml). The pooled organic phases were washed with wat r (20 ml). The product was

extracted fr m the pooled water phases with dichloromethane (50 ml) that after evaporation gave an oil.
^1H-n.m.r.($CDCl_3$); 1.26(d,6H);3.07(s,2H);3.6-3.8(m,12H); 4.11(s,2H);5.09(m,1 H)

8-(N-phtalimidoyl)-3,6-dioxa-octanol (3).
8-Chloro-3,6-dioxa-octanol (365 g, 2.2 mole, prepared from from triethylene glycol and $SOCl_2$) was dissolved in dimethyl formamide (400 ml) and potassium phtalimide (370 g, 2.0 mole) was added under stirring. After stirring for 18 hours at 110°C dimethyl formamide was distilled off at reduced pressure. The residue was suspended in toluene (1.5 l) at 40-50°C and potassium chloride was filtrated off. The product crystallizes at cooling (-10°C). A second fraction is available from the mother liquor by concentrating it and repeating the crystallization procedure.
^1H-n.m.r.($CDCl_3$); δ2.90(s,1H);3.51-3.58(m,2H);3.60-3.68(m,6H);3.73-3.78(t,2H);3.89-3.94(t,2H);7.70-7.89(m,4H).

Isopropyl 17-(N-phtalimidoyl)-3,6,9,12,15-pentaoxa-heptadecanoate (4).
A solution of pyridine (2.8 ml, 35 mmole) in dichloromethane (30 ml) was added dropwise under stirring at about -5°C to a solution of 8-(N-phtalimidoyl)-3,6-dioxa-octanol (3) (8.5 g, 36 mmole) and trifluoromethanesulfonic acid anhydride (10.2 g, 36 mmole) in dichloromethane. After about 30 minutes the organic phase was washed with 0.5 M hydrochloric acid and water. After drying (Na_2SO_4) and filtration isopropyl 8-hydroxy-3,6-dioxa-octanoate (1) (12 g, 48 mmole) and Na_2PO_4 (6.5, 46 mmole) were added, and the mixture was vigorously stirred for 20 hours at room temperature. The reaction mixture was filtrated and the filtrate evaporated. The residue was partiti ned between 1,1,1-trichloroethane and water. Evaporation of the organic phase r sulted in an oil (13 g).

^{1}H-n.m.r.($CDCl_3$); δ1.26(d,6H);3.58-3.76(m,18H);3.90(t,2H); 4.11(s,2H);5.09(m,1H);7.70-7.89(m,4H).

17-(N-phtalimidoyl)-3,6,9,12,15-pentaoxa-heptadecanoic acid (5).

Isopropyl 17-(N-phtalimidoyl)-3,6,9,12,15-pentaoxa-heptadecanoate (4) (13 g) was dissolved in tetrahydrofuran (50 ml) and hydrochloric acid (conc., 50 ml)). After 16 hours at room temperature the solution was diluted with water (200 ml) and tetrahydrofuran was removed at reduced pressure. The water phase was washed with toluene (1x) and extracted with dichloromethane (2x). Drying (Na_2SO_4) and evaporation of the organic phase resulted in the product in form of an oil (8.5 g)

^{1}H-n.m.r.($CDCl_3$): δ3.57-3.76 (m,18H);3.91(t,2H);4.11(s,2H); 4.8(br,2H);7.65-7.90(m,4H)

Isopropyl 17-amino-3,6,9,12,15-pentaoxa-heptadecanoate (6)

17-(N-phtalimidoyl)-3,6,9,12,15-pentaoxa-heptadecanoic acid (5) (8.5 g) was dissolved in 150 ml ethanol and 3 ml hydrazine hydrate. The solution was stirred at room temperature for 16 hours, whereupon HCl (100 ml, 3M) was added and the solution was then refluxed for 3 hours. After cooling to room temperature and filtration, pH was adjusted (pH 9, NaOH) and the filtrate was evaporated almost to dryness. Water was added and re-evaporation almost to dryness was carried out, whereupon the pH of the solution was adjusted (pH 4, HCl) followed by evaporation to dryness. The product was treated with isopropanol (100 ml) and acetyl chloride (2 ml) at room temperature during the night and evaporated. The residue was collected in water and extracted into dichloromethane at an alkaline pH (7-11). Evaporation resulted in the product (3.3 g).

^{1}H-n.m.r.(CH_3OD): δ1.26(d,6H);3.17(t,2H);3.65-3.80(m,18H); 4.16(s,2H);5.07(m,1H)

7,7,7-Triphenyl-3,6-dioxa-heptanol (7).

Triph nylmethyl chlorid (28 g, 0.1 mole) was added to a solution of pyridine (8 ml, 0.1 mole) in diethylene glycol (100 ml, 0.94 mole) and the mixture was stirred at 50°C for 20 hours. The crystals formed were filtrated off and washed with water. Yield 31 g. M.p. 103-105°C.
^{1}H-n.m.r.(CH_3OD): δ2.42(br,1H);3.25(t,2H),3.55-3.72(m,6H);7.20-7.32(m,9H);7.44-7.53(m,6H)

10,10,10-Triphenyl-3,6,9-trioxa-decanol (8).

Triphenylmethyl chloride (187 g, 0.67 mole) was added to a solution of pyridine (54 ml, 0.67 mole) in triethylene glycol (1,000 ml, 7.3 mole) and the mixture was stirred at 60°C for 16 hours. The product mixture was divided into 4 portions and each portion (=250 ml) was shaken with water (1,000 ml) and dichloromethane (250 ml). The organic phases were pooled and evaporated. Yield about 259 g.

13,13,13-Triphenyl-3,6,9,12-tetraoxa-tetradecanol (9).

Triphenylmethyl chloride (129 g, 0.46 mole) was added to a solution of pyridine (38 ml, 0.48 mole) in tetraethylene glycol (800 ml, 4.2 mole) and the mixture was stirred at 80°C for 20 hours. Water (800 ml) was added and the mixture was extracted with dichloromethane (3x200 ml). The pooled organic phases were washed with water (150 ml), whereafter the product was obtained as a syrup upon evaporation. Small amounts of the disubstituted and the unreacted materials were present in the product. Yield 200 g.

16,16,16-Triphenyl-3,6,9,12,15-pentaoxa-hexadecanol (10).

Triphenylmethyl chloride (2.8 g, 10 mmole) was added to a solution of pyridine (1 ml, 12 mmole) in pentaethylene glycol (25 g, 0.1 mole) and the mixture was stirred at 80°C for 2 hours. Wat r (100 ml) was added and the mixtur was extracted with dichlorom thane (40 ml). The water phase was evaporated by us of az otropic distilllation in the presence of t luene and thanol. The residu was treated

with pyridine (1 ml) and triphenylmethyl chloride (2.5 g) in the same manner as above. The same procedure was appli d once more. The three organic phases were pooled and evaporated. Small amounts of the disubstituted and of the unreacted materials were present in the product. Yield 10 g.

<u>19,19,19-Triphenyl-3,6,9,12,15,18-hexaoxa-nonadecanol (11).</u>
Triphenylmethyl chloride (9.7 g, 34 mmole) was added to a solution of pyridine (2.8 ml, 12 mmole) in hexaethylene glycol (100 g, 36 mole) and the mixture was stirred at 80°C for 3 hours. Water (400 ml) was added and the mixture was extracted with dichloromethane (150 ml). The water phase was treated once more as described in the example above, but the amounts and reaction conditions were as described in this example. The pooled organic phases were evaporated and washed with water (100 ml) and then extracted with dichloromethane (50 ml). Small amounts of the disubstituted and of the unreacted materials were present in the product. Yield 41.6 g.

<u>7,7,7-Triphenyl-3,6-dioxa-heptyl 4-methylbenzenesulfonate (12).</u>
p-Toluenesulfonyl chloride (5 g, 26 mmole) was added to a solution of 7,7,7-triphenyl-3,6-dioxa-heptanol (7) (3.5, 10 mmole) in pyridine (10 ml) . The mixture was stirred at room temperature for 30 minutes, whereupon water (1 ml) was added and the stirring continued for 10 minutes more. Dichloromethane (100 ml) was added and the mixture was extracted with hydrochloric acid (1 M) until the pyridine had been removed completely . Thereafter the organic phase was washed with $NaHCO_3$ (saturated). After drying (Na_2SO_4) and evaporation, the product crystallized from diethyl ether/hexane. Yield 2g.

10,10,10-Triphenyl-3,6,9-trioxa-decyl 4-methylbenzenesulfonate (13).

p-Toluenesulfonyl chloride (5 g, 26 mmole) was added to a solution of 10,10,10-triphenyl-3,6,9-trioxa-decanol (8) (4.8 g, 12 mmole) in pyridine (10 ml). The mixture was stirred at room temperature for 1 hour, whereupon water (1 ml) was added and the stirring continued for 10 minutes more. The mixture was diluted with dichloromethane (50 ml) and washed with 1 M hydrochloric acid (2x100 ml), water (50 ml) and $NaHCO_3$ (saturated, 50 ml). The organic phase was evaporated and the product was purified on silica gel (toluene:ethyl acetate, 19:1), whereupon the product crystallized from dichloromethane/ether. Yield 4.2 g. ^{1}H-n.m.r.($CDCl_3$): δ 2.20(s,3H);3.24(m,2H);3.60-3.80(m8H); 4.20(m,2H);7.20-7.90(m,19H).

8-(N-phtalimidoyl)-3,6-dioxa-octyl 4-methylbenzenesulfonate (14).

8-(N-phtalimidoyl)-3,6-dioxa-octanol (3) (82.2 g, 0.3 mole) was dissolved in pyridine 30 ml, 0.37 mole) and p-toluenesulfonyl chloride (56.9 g, 0.3 mole) was added during 1 hour and stirring at 0^{o}C. The mixture was then stirred at 10^{o}C for 16 hours. A solid cake had been formed and ice (0.5 kg) mixed with HCl (conc., 200 ml) was added and the mixture stirred at 50^{o}C for 4 hours, whereafter ethyl acetate (200 ml) was added. Filtration of the complete reaction mixture resulted in the product in solid form. Yield 97 g. ^{1}H-n.m.r.($CDCl_3$): δ 2.44(s,3H);3.52-3.73(m,8H);3.87(t,2H); 4.09(q,2H);7.31-7.86(m,8H)

17-(N-phtalimidoyl)-3,6,9,12,15-pentaoxa-heptadecanol (15).

10,10,10-Triphenyl-3,6,9-trioxa-decanol (8) (0.78 g, 2 mmole) dissolved in dim thyl formamide (15 ml) was add d dropwise t sodium hydrid (80%, 0.24 g, 8 mmole, washed with 2x hexane). The mixture was warmed to 30^{o}C for 30 minut s wh reaft r 8-(N-phtalimidoyl)-3,6-dioxa-octyl 4-

methylbenzenesulfonate (14) (0.87 g, 2 mmole) was added in solid form. After stirring during the night at room temperature, acetic acid anhydride was added (5 ml), and the mixture was allowed to stand for three hours more at room temperature. Water (2 ml) was added and after 30 minutes the product was partitioned between toluene (25 ml) and a solution of $NaHCO_3$ (50 ml). The toluene phase was evaporated and contained about 1 g substance that was collected in dichloromethane and treated with 0.2 ml trifluoroacetic acid and about 0.2 ml water at room temperature for 10 minutes. The reaction mixture was then evaporated. The product was purified on a silica column (chloroform:methanol, 19:1). Yield about 0.7 g.
^{1}H-n.m.r.($CDCl_3$): δ 3.59-3.67(m,20H);3.71-3.75(m,4H); 3.90(t,2H);7.71-7.86(m,4H)

<u>tert-Butyl 17-(N-phtalimidoyl)-3,6,9,12,15-pentaoxa-heptadecanoate (16).</u>
Pyridinium dichromate (0.64 g, =4 eq.), acetic acid anhydride (1 ml = 20 eq.) and t-butyl alcohol (1 ml = 30 eq.) were added in the order given to a solution of 17-(N-phtalimidoyl)-3,6,9,12,15-pentaoxa-heptadecanol (15) (190 mg) in dichloromethane (5 ml). The reaction mixture was stirred at room temperature for 3 hours, whereafter ethyl acetate (25 ml) was added. After 10 minutes the liquid was allowed to pass through a column containing silica gel (= 5 cm x 5 cm O) and the column was eluted with more ethyl acetate. After evaporation the residue was purified on a silica gel column (chloroform:methanol, 19:1) Yield about 0.1 g.
^{1}H-n.m.r.($CDCl_3$): δ1.47(s,9H)·3.58-3.76(m,18H);3.90(t,2H); 4.02(s,2H);7.70-7.87(m,4H)

<u>14,14,14-Triphenyl-4,7,10,13-tetraoxa-tetradecene (17).</u>
Allyl br mide (2 ml, 1.1 eq.) and 10,10,10-triphenyl-3,6,9-tri xa-decanol (8) (8.5 g, 22 mmole) wer diss lv d in dimethyl formamide (25 ml) and added dropwise into sodium

hydrid (1 g) at 0°C during 30 minutes. Aft r stirring for three hours at room temperature, methanol was added until the solution became transparent. Toluene (100 ml) and water (100 ml) were added and the reaction mixture was extracted. The toluene phase was washed with a saturated salt solution and was then dried (Na_2SO_4). The product was obtained as a syrup (10 g) after evaporation of the solvent.

3,6,9-Trioxa-11-dodecen-1-ol (18).

14,14,14-Triphenyl-4,7,10,13-tetraoxa-tetradecene (17) (5.5 g, 13 mmole) was dissolved in dichloromethane (50 ml) whereafter trifluoroacetic acid (1.2 ml, 15.6 mmole) and water (1 ml, 55 mmole) were added and the reaction mixture stirred vigorously for 10 minutes. The product (1.5 g) was obtained after purification on a silica gel column ($CHCl_3$:MeOH, 9:1).

3,6,9,12,15,18-Hexaoxa-20-heneicosen-1-ol (19).

3,6,9-Trioxa-11-dodecen-1-ol (18) (2.07 g, 10.9 mmole) and 10,10,10-triphenyl-3,6,9-trioxa-decyl 4-methyl-benzenesulfonate (13) (5.95 g, 10.9 mmole) were dissolved in dimethyl formamide and added dropwise under stirring to sodium hydride for 10 minutes at room temperature. After 2 hours dichloromethane and water were added. The phases were separated, and trifluoroacetic acid (1 ml, 13 mmole) and water (1 ml, 55 mmole) were added to the organic phase. The mixture was evaporated to dryness after vigorous stirring for one hour. Methanol (70%) was added whereupon triphenyl methanol (2 g) was filtrated off and the solvent evaporated. The product (2.5 g) was obtained after purification on a silica gel column ($CHCl_3$:MeOH, 97:3). ^{1}H-n.m.r.($CDCl_3$): δ3.56-3.74(m,24H);4.00(m,2H);5.20(m,2H); 5.89(m,1H)

3,6,9,12,15,18,21-Heptaoxa-23-tetracosenoic acid (20).

Br moacetic acid (0.15 g, 1.1 mmole) and sodium hydride (0.15 g, 5 mmole) were added t a solution of

3,6,9,12,15,18-Hexaoxa-20-heneicosen-1-ol (**19**) (0.33 g, 1 mmole) in tetrahydrofuran. The mixture was stirred at room temperature for three hours, whereupon water (50 ml) was added in order to degrade the excess of reagent and pH was adjusted to pH 1 (HCl). The mixture was washed with diethyl ether (50 ml) and was then extracted with dichloromethane (2x50 ml). Drying (Na_2SO_4) and evaporation of the solvent gave the product (0.26 g).

<u>23-(N-tert-butoxycarbonylamino)-3,6,9,12,15,18,21-heptaoxa-tricosanoic acid (**21**).</u>

Ozone was bubbled slowly through a solution of 3,6,9,12,15,18,21-heptaoxa-23-tetracosenoic acid (**20**) (0.21 g, 0.55 mmole) in methanol (30 ml) for 30 minutes at approximately -60°C. The mixture was allowed to stand for 30 minutes, and then air was bubbled through the solution for 30 minutes, whereupon dimethyl sulfide (50 μl, 0.65 mmole) dissolved in methanol (5 ml) was added dropwise. The solution was tempered to room temperature during one hour, whereupon a solution of ammonium chloride (0.2 g, 3.7 mmole) in water (5 ml) and sodium cyano borohydride (0.2 g, 3.1 mmole) was added. After 16 hours at room temperature the pH of the solution was adjusted to about 1 (HCl), whereupon the solvent was removed by distillation. The dry solid phase was extracted with dichloromethane that then was evaporated. Dissolution in sodium hydroxide (1 M), extraction with dichloromethane and evaporation resulted in an oil (0.25 g). A pure product was obtained by preparing the tert-butoxycarbonyl derivative of the amino function; 0.17 g of the oil and sodium hydroxide (0.4 g) were dissolved in water (3 ml) and di-tert-butyl dicarbonate (0.12 g) dissolved in dioxane (5 ml) were added. After stirring for 16 hours the dioxane was evaporated and the water phase was washed with n-hexane (2x10 ml) and pH was adjusted to about 4 (HCl), wher upon the mixtur was extracted with dichloromethane (5x50 ml). Drying (Na_2SO_4) and evaporati n of the solv nt gave an il

that was purified on a silica g l column ($CHCl_3$:MeOH:AcOH, 45:4:1). The yield was 0.15 g.
^{1}H-n.m.r.(D_2O, 500 MHz): δ 1.31(s,9H);3.14(t,2H);3.48(t,2H); 3.59(m,24H);3.84(s,2H)

11-(N-phtalimidoyl)-3,6,9-trioxa-undecanoic acid (22).
Sodium hydride (3.8 g, 80%, 130 mmole) and bromoacetic acid (7.6 g, 55 mmole) dissolved in tetrahydrofuran (5 ml) were added in the order given to a solution of 8-(N-phtalimidoyl)-3,6-dioxa-octanol (3) (10.1 g, 36 mmole) in tetrahydrofuran (150 ml). After stirring for four hours at room temperature acetic acid anhydride (40 ml) was added and the mixture was stirred for 18 hours more, whereafter the mixture was filtrated and evaporated in the presence of some water. Purification of the product on silica gel ($CHCl_3$:MeOH, 3:2) resulted in the product. Yield 1.2 g.

Methyl 11-amino-3,6,9-trioxa-undecanoate (23).
Hydrazine hydrate (0.7 ml, 14 mmole) was added to a solution of 11-(N-phtalimidoyl)-3,6,9-trioxa-undecanoic acid (22) (1.2 g, 3.5 mmole) in ethanol (25 ml) and the mixture was stirred at room temperature for 18 hours, whereupon hydrochloric acid (3.7 ml, conc.) and water were added. The mixture was refluxed for two hours and was then evaporated almost to dryness whereafter water (25 ml) was added and pH adjusted to about 9 (NaOH). After evaporation to dryness methanol (200 ml) and acetyl chloride (2 ml) were added and the mixture was stirred at room temperature for 66 hours. The methyl ester formed was purified on a silica gel column.
^{1}H-n.m.r.(D_2O): δ 3.17(t,2H);3.65-3.80(m,13H);4.20(s,2H);

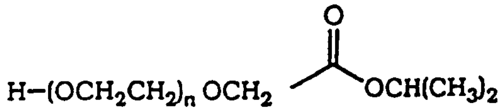
3,6,9,12,15,18,21,24,27-nonaoxa-29-tricontenoic acid (24).
Sodium hydride (50 mg, 1.6 mmole) was added t a solution of 3,6,9,12,15,18-hexaoxa-20-h neicosen-1-ol (19) (0.6 g, 1.9 mmol) and 7,7,7-triphenyl-3,6-dioxa-heptyl 4-methylb nzen sulfonate (12) (0.9 g, 1.8 mmole) in dimethyl-

formamide (10 ml). The mixture was stirred at room temperature for 56 hour, whereupon wat r (10 ml) and dichloromethane (15 ml) were added. The mixture was shaken and the organic phase was evaporated and purified on a silica gel column ($CHCl_3$:MeOH, 9:1). The product was dissolved in dichloromethane (20 ml) and trifluoroacetic acid (3 drops) and water (5 drops) were added, whereafter the mixture was stirred for 4 hours and evaporated. Extraction of the residue with methanol:water (70:30) and filtration gave upon evaporation an oil that was dissolved in tetrahydrofuran (10 ml) to which sodium hydride (about 5 eq.) and bromoacetic acid (about 2 eq.) were added, whereafter the mixture was stirred at room temperature for 16 hours. Water was added in order to destroy excess of sodium hydride, and the mixture was evaporated and then purified on a silica gel column ($CHCl_3$:MeOH, 3:1). Yield 0.14 g.
^{1}H-n.m.r.($CDCl_3$): δ2.89(s,2H);2.97(s,2H);3.50-3.78(m,30H);4.02(d,2H);5.20(m,2H);5.91(m,1H)

<u>29-(N-tert-butoxycarbonylamino)-3,6,9,12,15,18,21,24,27-nonaoxa-29-nonacosanoic acid (25).</u>
Ozon was bubbled smoothly through a solution of 3,6,9,12,15,18,21,24,27-nonaoxa-29-tricontenoic acid (24) (0.11 g, 0.23 mmole) in methanol (25 ml) for 30 minutes at about 70°C. 30 minutes later, air was bubbled for 30 minutes through the solution, whereafter dimethyl sulfide (50 μl, 0.65 mmole) was added. The solution was tempered to room temperature during a period of one hour, whereafter a solution of ammonium hydrochloride (0.1 g, 1.9 mmole) in water (5 ml) and sodium cyano borohydride (0.1 g, 1.6 mmole) were added. After 16 hours at room temperature pH was adjusted to about 1 (HCl), and the solvent was evap rated. The dry material was extracted with dichloromethane which was then evap rat d. The residue was an oil (0.1 g). A pure product was btained by preparing a tert-butoxycarbonyl derivative f the amino functi n; the

il and sodium hydroxide (0.4 g) were dissolved in water (3 ml) and di-t rt-butyl dicarbonate (0.16 g) dissolved in dioxane (5 ml) was added. After stirring for 16 hours the dioxane was evaporated and the water phase was washed with n-hexane (2x10 ml) and the pH was adjusted to about 4 (HCl), whereupon the product was extracted with dichloromethane.

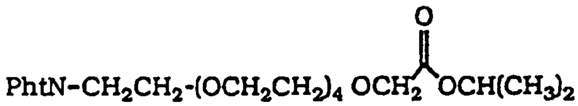
FORMULAE OF SYNTHESIZED AMINO-PEG-CARBOXYLIC ACIDS



1 n=2
2 n=3

PhtN—CH_2CH_2—$(OCH_2CH_2)_2$ OH

3



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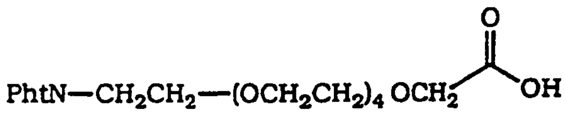
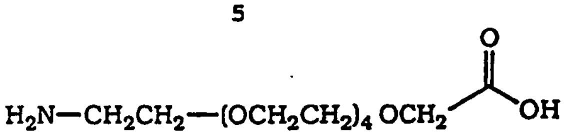


5



6

Tr—$(OCH_2CH_2)_n$ OH

7 n=2
8 n=3
9 n=4
10 n=5
11 n=6

Tr—$(OCH_2CH_2)_n$ OTs

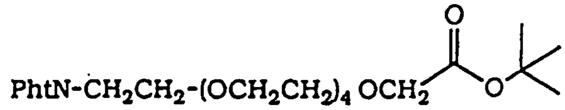
12 n=2
13 n=3

PhtN—CH_2CH_2—$(OCH_2CH_2)_2$ OTs

14

PhtN—CH_2CH_2—$(OCH_2CH_2)_4$ OCH_2CH_2OH

15



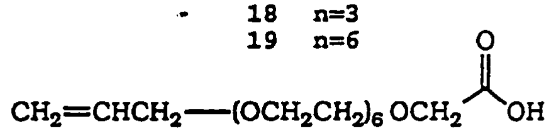
16

CH_2=$CHCH_2$—$(OCH_2CH_2)_3$ OTr

17

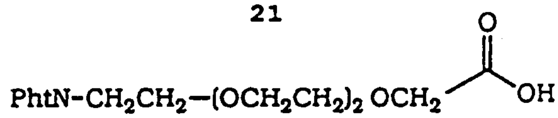
CH_2=$CHCH_2$—$(OCH_2CH_2)_n$ OH

18 n=3
19 n=6

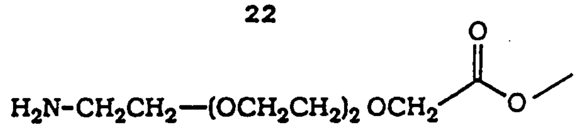


20

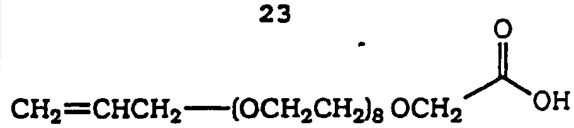
21



22



23



24

25

PREPARATION OF BIFUNCTIONAL REAGENTS AND COUPLING PRODUCTS

Structural formulae are set forth on a separate page.

Example 1. **Preparation of N-hydroxysuccinimide ester of 17-iodoacetylamino-3,6,9,12,15 pentaoxaheptadecanoic acid**

A. Preparation of 17-iodoacetylamino -3,6,9,12,15-pentaoxaheptadecanoic acid (A)

Isopropyl 17-amino-3,6,9,12,15-pentaoxaheptadecanoate (see part I of the experimental part) (1.1 g, 3.2 mmole) was dissolved in 3 ml of 1 M sodium hydroxide solution and left at room temperature for 30 min. 1.5 ml of 6 M hydrochloric acid was added and the mixture was evaporated to dryness. The residue was taken up in dichloromethane and filtered to give 545 mg of 17-amino-3,6,9,12,15-pentaoxaheptadecanoic acid after evaporation of the solvent. 460 mg (1,39 mmoles) of this compound were dissolved in 10 ml of borate buffer pH 8.4. The solution was deaerated with nitrogen gas. A solution of 432 mg (1.52 mmoles) of N-succinimidyl 2-iodoacetate in 5 ml of dioxane was added dropwise during 1 min pH was kept at 8.4 by addition of 5 M NaOH. The reaction solution was stirred for 15 min during inlet of nitrogen gas. According to thin layer chromatography (eluent: CH_2Cl_2-MeOH 60:35) the reaction was completed in some few minutes. After 15 min the pH of the reaction solution was adjusted to 3 and the s lution was frozen and lyophilized. The reaction mixture was fractionated on a reversed phase column PEP-RPC HR 30/26 (Pharmacia Biosystems AB) using a gradient of 0-13 % acetonitrile with 0.1 % trifluoroacetic acid followed by isocratic separation at 13 % acetonitrile, 0.1 % TFA. Fractions from the

desired peak were pooled and lyophilized giving 351 mg of 17-iodoacetylamino-3,6,9,12,15-pentaoxa-heptadecanoic acid (A). Yield: 76 %.

The structure of the product was established by the aid of its NMR spectrum. ^{1}H NMR spectrum (D_2O) expressed as δ-values:

$IC\underline{H}_2C$ 4.23 s, $OC\underline{H}_2COH$ 3.76 s
O O
$-OC\underline{H}_2C\underline{H}_2O-$ 3.71-3.76, $-NHCH_2C\underline{H}_2O-$ 3.65 t,
$-NHC\underline{H}_2CH_2O-$ 3.41

B. <u>Preparation of N-hydroxysuccinimide ester of 17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoic acid</u> (B)

Hydroxysuccinimide (4.5 mg, 39 µmole) was weighed in the reaction vial. 17-Iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoic acid (A) (18.3 mg, 39 µmole) was dissolved in 0.55 ml dried dioxane and added to the reaction vial. The vial was deareated with nitrogen gas and then a solution of 8.0 mg (39 µmole) dicyclohexylcarbodiimide in 0.15 ml of dried dioxane was added dropwise to the reaction vial. The vial was filled with nitrogen gas, closed and placed in the dark. The reaction solution was stirred for 3.5 h. The precipitate formed was removed by filtration. The percentage formed product B in the filtrate was determined by NMR-analysis to be 89 %.

Example 2. Preparation of (17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoylamino)-immunoglobulin (C)

A. Monoclonal antibody Mab C215

A monoclonal antibody of immunoglobulin class IgG2a (Mab C215) (34 mg, 0.218 µmole) dissolved in 17.7 ml of 0.1 M borate buffer pH 8.1 containing 0.9 % sodium chloride was added to a reaction vial. 146 µl of a dioxane solution containing 3.6 mg (6.4 µmole) of N-hydroxysuccinimide ester of 17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoic acid (B) was injected into the buffer solution and the reaction was completed during stirring for 25 min. at room temperature. The reaction vial was covered with folie to exclude light. Excess of reagent B was removed by fractionation on a Sephadex G 25 K 26/40 column using 0.1 M phosphate buffer pH 7.5 containing 0.9 % sodium chloride as eluent. Fractions containing the desired product C were pooled. The solution (22 ml) was concentrated in an Amicon cell through a YM 30 filter to 8 ml. The concentration and degree of substitution were determined with amino acid analysis to be 4.7 mg/ml and 18 spacer per Mab C215 respectively.

B. Monoclonal antibody Mab C242

A monoclonal antibody (Mab C242) of the immunoglobulin class IgG 1 was reacted with 15, 20 and 22 times molar excess of N-hydroxysuccinimide ester of 17-iodoacetyl-amino-3,6,9,12,15-pentaoxaheptadecanoic acid (B) respectively according to the procedure described in exampl 2.A giving n na, dodeca and t tradeca(17-iodoacetylamino)-3,6,9,12,15-pentaoxaheptadecanoylamino)-Mab C242. (C)

C. Monoclonal antibody Mab C

A monoclonal antibody (Mab C) of the immunoglobulin class IgG 2a was reacted with 14 and 18 times molar excess of N-hydroxysuccinimide ester of 17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoic acid (B) respectively according to the procedure described in example 2A giving tetra and hepta(17-iodo-acetylamino-3,6,9,12,15-pentaoxa-heptadecanoylamino)-Mab C. (C)

Example 3. **Preparation of 2-mercaptopropionylamino-Eu^{3}-labelled-staphylococcal enterotoxin A (SEA)**

A. Preparation of Eu^{3+} labelled SEA (D)

SEA (freezed dried product from Toxin Technology Inc.) (2 mg, 72 nmole) was dissolved in 722 µl milli-Q water and added to a 15 ml polypropylene tube. 100 µl of 0.1 M borate buffer pH 8.6 was added and then 2160 nmoles of Eu^{3+}-chelate reagents (Pharmacia Wallac Oy) in 178 µl of milli-Q. The reaction was completed at room temperature over night. Excess reagent was removed by fractionation of the reaction solution on a Sephadex G 25 PD 10 column (Pharmacia Biosystems AB) using 0.1 M phosphate buffer pH 8.0 as eluent. Fractions with the desired product D were pooled. The solution (3 ml) was concentrated in an Amicon cell through an YM5 filter to a volume of 0.8 ml. The concentration was determined with amino acid analysis to be 1.7 mg/ml. The degree of substitution was determined by comparing with a $EuCl_3$ standard solution to be 0.8 Eu^{3+} per SEA.

B1. Preparation of 3-(2-pyridyldithio)propionylamino Eu^{3+} labelled SEA (E) and 3-mercaptopropionylamino Eu^{3+} labelled SEA (F)

Eu^{3+}-SEA (1.24 mg, 44.8 nmoles) in 0.75 ml of 0.1 M phosphate buffer pH 8.0 was added to a 15 ml polypropylene tube. 35 µl (180 nmole) of a solution of 1.6 mg of N-succinimidyl 3-(2-pyridyldithio)-propionate in 1 ml of ethanol was added to the tube and the reaction solution was stirred for 30 min at room temperature. The obtained product E was not isolated before being reduced to product F.

To the reaction solution from above were added 20 µl of 0.2 M Eu^{3+}-citrate solution and 50 µl of 2 M acetic acid to adjust the pH to 5. Thereafter a solution of 3.1 mg of dithiotreitol (Merck) in 0.1 ml of 0.9 % sodium chloride was added and the reaction solution was stirred for 20 min at room temperature. Thereafter the total volume was adjusted to 1 ml by addition of 50 µl of 0.9 % sodium chloride solution. The reaction solution (1 ml) was placed on a Sephadex G25 NAP-10 column (Pharmacia Biosystems AB) and desired product F was eluted by addition of 1.5 ml of 0.1 M phosphate buffer pH 7.5 containing 0.9 % sodium chloride. The eluted product F was collected in a 15 ml polypropylene tube and immediately used in the synthesis of product G to avoid reoxidation to a disulfide compound.

B2. Preparation of 2-mercaptopropionylaminostaphylococcal enterotoxin A (SEA) (F2)

Native SEA (freeze dried product from Toxin Technology Inc) or recombinant prepared SEA (rSEA) was reacted with 2 times molar excess of N-succinimidyl 3-(2-pyridyldithio)-propionate according to the procedure described in example 3B1.

The degree of substitution was determined with UV-analysis according to Carlsson et al (Biochem. J. 173(1978)723-737) to be 1.9 mercaptopropionyl group per SEA.

Example 4. Preparation of the SEA-monoclonal antibody conjugate (G1 och G2)

A. Conjugates between Eu^{3+}-SEA and Mab C215 (G1)

To the solution of 4-mercaptopropionylamino Eu^{3+} labelled SEA (F) described in example 3B was added 1.2 ml of a solution of octadeca(17-iodoactylamino-3,6,9,12,15-pentaoxaheptadecanoylamino)Mab C215 (C) (4 mg) in 0.1 M phosphate buffer pH 7.5 containing 0.9 % sodium chloride. The reaction was completed by standing at room temperature over night. Unreacted iodinealkyl groups were then blocked by addition of 5 µl (1.2 µmole) of a solution of 20 µl mercapto-ethanol in 1 ml of water. The reaction solution was left for 4 h at room temperature and then filtrated. The filtrate was then fractionated on a Superose 12 HR 16/50 column (Pharmacia Biosystems AB) using as eluent 0.002 M phosphate buffer pH 7.5 containing 0.9 % sodium chloride. Fractions with the desired product G were pooled and analysed. The protein content was 0.22 mg/ml determined by amino acid analysis. The degree of substitution was one SEA per IgG determined by Eu^{3+} determination. The product was also studied for immunostimulating properties and antib dy binding capacity.

By increasing the amount of compound (F) in relation to compound (C) high r degr of substitution was obtained.

B. Conjugates between rSEA and Mab C215 (G2)

Octadeca (17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoylamino)Mab C215 (C) was reacted with 1.8 times molar excess of 2-mercaptopropionyl-amino-rSEA (F2) according to the procedure described in example 4A. The composition of the conjugate was analysed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on Phast-Gel™ gradient 4-15 and the bonds were scanned with Phast IMAGE (Pharmacia Biosystems AB). The conjugate obtained was composed of 6% Mab C215 with three SEA, 15% with two SEA, 28% with one SEA and 51% unsubstituted Mab C215.

In another experiment 2.7 times molar excess of F2 was used giving a conjugate with the composition 15% Mab C215 with three SEA, 25% with two SEA, 34% with one SEA and 26% of unsubstituted Mab C215.

C. Conjugates between rSEA and Mab C242

C1. Tetradeca(17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoylamino)Mab C242 (C) was reacted with 3.2 times molar excess of 2-mercaptopropionyl-amino-rSEA (F2) according to the procedure described in example 4A. The composition of the conjugate was analysed as described in example 4 B and was found to be 4% Mab C242 with four SEA, 12% with three SEA, 28% with two SEA, 36% with one SEA and 20% of unsubstituted Mab C242.

The same r action was run but the reaction product was treated 4 h with 0.2 M hydroxylamin before column fractionation t remove unstable bonds between Mab C242 and the spacer and between SEA and the mercaptopropionyl group. The conjugate formed had the composition 1% Mab C242 with four SEA, 12% with three

SEA, 27% with two SEA, 36% with one SEA and 24% of unsubstituted Mab.

C2. Dodeca(17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoylamino)Mab C242 (C) was reacted with 3 times molar excess of 2-mercaptopropionylamino-rSEA (F2) according to the procedure described in example 4A. The conjugate obtained had the composition 6% Mab C242 with three SEA, 26% with two SEA, 36% with one SEA and 31% unsubstituted Mab C242.

C3. Nona(17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoylamino)Mab C242 (C) was reacted with 3 times molar excess of 2-mercaptopropionylamino-rSEA (F2) according to the procedure described in example 4A. The conjugate obtained had the composition 13% Mab C242 with two SEA, 39% with one SEA and 46% unsubstituted Mab C242.

D. <u>Conjugates between rSEA and Mab C</u>

D1. Hepta(17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoylamino)Mab C was reacted with 5.4 times molar excess of 2-mercaptopropionylamino-rSEA (F2) according to the procedure described in example 4A. The composition of the conjugate was analysed as described in example 4B and was found to be 15% with four SEA, 24% with three SEA, 29% with two SEA, 19% with one SEA, 3% unsubstituted Mab C and 10% in a dimeric form.

Th same reaction was run with 0.2 M hydroxylamine present to remove unstable bonds b tw en Mab C and spacer and b tw n SEA and the mercaptopropionyl group. The conjugate formed had the following c mposition 11% Mab C with thr e SEA, 24% with two

SEA, 30% with one SEA, 18% of unsubstituted Mab C and 17% in a dimeric form.

D2. Tetra(17-iodoacetylamino)-3,6,9,12,15-pentaoxaheptadecanoylamino)Mab C was reacted with 5.7 times molar excess of 2-mercaptopropionylamino-rSEA (F2) according to the procedure described in example 4B. The conjugate had the following compositon 8% Mab C2 with four SEA, 18% with three SEA, 30% with two SEA, 26% with one SEA, 5% of unsubstituted Mab C and 12% in a dimeric form.

Example 5. Coupling of the peptide sequence 145-165 derived from the human alloantigen HLA-A2.1 to the monoclonal antibody Mab C215 (H)

Octadeca(17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoylamino)-immunoglobylin G2a (C) (5.4 mg, 34.6 nmole) dissolved in 1.6 ml of 0.1 M phosphate buffer pH 7.5 containing 0.9 % sodium chloride was added to a 5 ml Reacti vial. The solution was deaerated by nitrogen gas and then the HLA-A2.1 peptide sequence 145-165 HisLysTrpGluAlaHisValAlaGluGlnLeuArgAlaTyrLeuGluGlyThrCysVal (2.5 mg, 0.8 µmole) was added in solid state in small portions during stirring to the solution. The pH of the reaction solution was chequed to be 7.4. Before closing the vial more nitrogen gas was bubbled through the solution. The vial was covered with folie and the reaction solution was stirred over night at room temperature. To block unreacted iodinealkyl groups 10.5 µl (1.5 µmole) of a solution of 10 µl mercaptoethanol in 1 ml water was added and the reaction solution was stirred for 4 h. The reaction soluti n was filtered and fractionated on a Superose 12 HR 10/50 column (Pharmacia Biosystems AB) using 2 mM phosphat buffer pH 7.5 containing 0.9 % sodium chloride as eluent. Fractions with the desired product (H) were pooled. The protein concentration and

degree of modification were determined by amino acid analysis to be 176 µg protein per ml and 11 peptides per IgG.

In a similar synthesis the peptide was added in an amount of 5 mg (1.7 µmole) giving a product with 17 peptides per IgG.

Example 6. **Coupling of the peptide sequence 93-113 derived from the human alloantigen HLA-A2.1 to the monoclonal antibody Mab C215 (1)**

Tridecane (17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoylamino)-immunoglobulin G2a (4 mg, 25.6 nmole) dissolved in 1.6 ml 0.1 M phosphate buffer pH 7.5 containing 0.9 % sodium chloride was added to a 5 ml Reacti vial. (This compound was prepared similar to compound C, Example 2A,using less excess of the reagent B). The solution was deaerated by nitrogen gas. The HLA-A2.1 peptide MetTyrGlyCysAspValGlySerAspTrpArgPheLeuArgGlyTyr (4.7 mg, 2.1 µmole) was suspended in 0.2 ml of acetonitrile and dissolved by addition of 0.1 ml of 0.1 M phosphate buffer pH 7.5 containing 0.9 % sodium chloride. This solution was added dropwise to the Reacti vial. pH was chequed to be 7.5. Nitrogen gas was bubbled through the reaction solution before the vial was closed. The vial was covered with folie and the reaction solution was stirred over night. To block unreacted iodinealkyl group 10 µl (1.4 µmole) of a solution of 10 µl mercaptoethanol in 1 ml water were added. The reaction solution was stirred for another 4 h, filtered and then fractionated on a Superose 12 HR 10/50 column using 2 mM phosphate buff r pH 7.5 containing 0.9 % sodium chlorid as luent. Fractions with the desired product (I) were pool d. The protein concentration and d gr e of modification wer determined by amino acid analysis to be 196 µg protein per ml and 7 peptides per IgG respectiv ly.

Example 7. Preparation of N-hydroxysuccinimide ester of 17-[3-(2-pyridyldithio)propionylamino]-3,6,9,12,15-pentaoxaheptadecanoic acid (K)

A. Preparation of 17-[3-(2-pyridyldithio)propionylamino]-3,6,9,12,15-pentaoxaheptadecanoic acid (J)

17-Amino-3,6,9,12,15-pentaoxaheptadecanoic acid (66 mg, 0.2 mmole) was dissolved in 3.5 ml of 1 M borate buffer pH 8.4. The pH decreased to 8.1. N-Succinimidyl 3-(2-pyridyldithio)-propionate (69 mg, 0.22 mmole) dissolved in 0.8 ml of dioxane was added to the above solution. The pH of the reaction solution decreased to 7.6. The reaction was completed in 10 min which was shown by thin-layer chromatography (Eluent: CH_2Cl_2-MeOH 60:35).

After 30 min the pH of the reaction solution was adjusted to 4.5 with 5 M hydrochloric acid and the solution was frozen and lyophilized. The reaction mixture was fractionated on a reversed phase column PEP RPC HR 16/10 (Pharmacia Biosystems AB) using a gradient of 0.17 % acetonitrile with 0.1 % TFA followed by isocratic separation at 17 % acetonitrile with 0.1 % TFA. Fractions with the desired compound J were pooled and lyophilized. The fractionation was repeated 13 times. Yield: 39 mg.

The structure of the product J was established by the aid of its NMR spectrum.

B. Preparation of N-hydroxysuccinimide ester of 17-[3-(2-pyridyldithio)propionylamino]-3,6,9,12,15-pentaoxaheptadecanoic acid (K)

Hydrosuccinimide (1.87 mg, 16.3 µmole) was weighed in a 5 ml Reacti vial. 17-[3-(2-pyridyldithio)-propionylamino]-3,6,9,12,15-pentaoxaheptadecanoic acid

(J) (8.0 mg, 16.4 µmole) was dissolved in 0.5 ml of dried dioxane and added to the vial. Nitrogen gas was bubbled through the solution. A solution of 6.8 mg (32.8 µmole) of dicyclohexylcarbodiimide in 200 ml of dioxane was added to the Reacti vial and more nitrogen gas was bubbled through the reaction solution before closing the vial. The reaction was allowed to occur during 24 h and the precipitate formed was removed by filtration. NMR analysis of the filtrate showed that the reaction was almost completed to compound K.

<u>Example 8</u>. **Preparation of di(17-[3-(2-pyridyldithio)propionylamino]-3,6,9,12,15-pentaoxaheptadecanoylamino)immunoglobulin G1 (L)**

A monoclonal antibody of immunoglobulin class IgG1 (Mab C242) (6 mg, 38 mmole) dissolved in 2.23 ml of 0.1 M borate buffer pH 8.0 containing 0.9 % NaCl was added to a 5 ml Reacti vial. The solution was diluted with 0.77 ml of the above buffer to a final concentration of 2 mg protein per ml. A solution of N-hydroxysuccinimide ester of 17-[3-(2-pyridyldithio)propionylamino]-3,6,9,12,15-pentaoxaheptadecanoic acid (K) (0.26 mg, 447 nmole) dissolved in 100 µl of dioxane was injected into the solution in the Reacti vial. The reaction solution was stirred for 25 min at room temperature and then placed in the refrigerator over night. The reaction solution was fractionated on a Superdex 75 HR 10/30 column (Pharmacia Biosystems AB) using 0.1 M phosphate buffer pH 7.5 containing 0.9 % NaCl as eluent. Fractions with the desired product (L) were pooled (7 ml) and concentrated in an Amicon cell through an YM 30 filter to 1.5 ml. The concentration and degr of substitution w r d termined with amino acid analysis to be 3.51 mg protein per ml and 2 spacer per IgG respectiv ly.

Example 9. **Crosslinking of two monoclonal antibody molecules to product (N)**

A. Preparation of di(17-[3-thiopropionylamino]-3,6,9,12,15-pentaoxaheptadecanoylamino)-immunoglobulin G1 (M)

Di(17-[3-(2-pyridyldithio)propionylamino]-3,6,9,12,15-pentaoxaheptadecanoylamino)immunoglobulin G1 (L) (2.5 mg, 16 mmole) dissolved in 0.7 ml 0.1 M phosphate buffer pH 7.5 containing 0.9 % sodium chloride was added to an Ellenman tube. pH was adjusted to 4.7 with 1 M acetic acid. Thereafter 100 µl (2.3 mg) of a solution of 6.9 mg of dithiotreitol in 300 µl of 0.9 % sodium chloride was added. The reaction solution was standing at room temperature for 25 min and then desalted on a Sephadex G25 NAP 10 column (Pharmacia Biosystems AB). 0.1 M phosphate buffer pH 7.5 containing 0.9 % sodium chloride and 2 mg EDTA per ml was used as eluent. The product M was eluted in a volume of 1.5 ml and immediately used in the synthesis of product (N) to avoid reoxidation of the free mercapto groups.

B. Preparation of the crosslinked monoclonal antibody (N)

To a 5 ml Reacti vial were added 0.7 ml (1.25 mg) of the solution of (17-[3-thiopropionylamino]-3,6,9,12,15-pentaoxaheptadecanoylamino)immuno-globulin G1 (M) described in example 9A and 0.35 ml of a solution of tri(17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoylamino)immunoglobulin G1 (1.26 mg). (This compound was pr pared similar to compound C using less xcess of the r agent B, and the monoclonal antibody Mab C242).

Nitrogen gas was bubbled through the reaction solution. Thereafter the vial was closed, covered with folie to avoid light and left over night at room temperature. Unreacted iodinealkyl groups were blocked by addition of 2.5 µl (0.6 µmole) of a solution of 20 µl of mercaptoethanol in 1 ml water. The reaction solution was standing at room temperature for 6 h and then fractionated on Superose 12 HR 10/30 column (Pharmacia Biosystems AB) using 5 mM phosphate buffer pH 7.5 containing 0.9 % sodium chloride as eluent. Fractions with the dimeric product (N) were pooled.

Example 10. **Preparation of [17-(3-mercaptopropionylamino)-3,6,9,12,15-pentaoxaheptadecanoylamino]-rSEA (P)**

A. Preparation of 17-[3-(2-pyridyldithio)propionylamino]-3,6,9,12,15-pentaoxaheptadecanoylamino)-rSEA (O)

A solution of N-hydroxysuccinimide ester of 17-[3-(2-pyridyldithio)propionylamino]-3,6,9,12,15-pentaoxaheptadecanoic acid (K) (0.53 mg (896 nmoles) in 43 µl of dioxane) was injected into a solution of 3.67 mg (128 nmoles) of rSEA in 1 ml of 0.1 M phosphate b fer pH 7.5 containing 0.9% of sodium chloride. The reaction was completed in 30 min at room temperature. 100 µl was taken for analysation of degree of substitution. The rest was stored frozen until it was reduced to product P.

The degree of substitution was determined by desalting 100 µl of the reaction solution on a Sephadex G50 NICK column (Pharmacia Biosystems AB) and analysing the eluate with UV-spectroscopy according to Carlsson et al (Biochem. J. 173(1978)723-737). 2.7 spacers were coupled to rSEA.

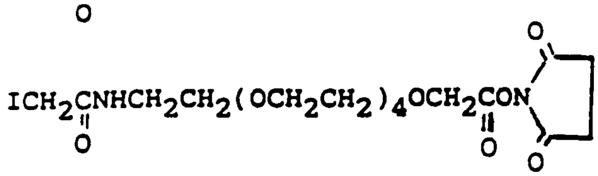
B. Preparation of [17-(3-mercaptopropionylamino)-3,6,9,12,15-pentaoxaheptadecanoylamino]-rSEA (P)

The pH of the reaction solution with product O (0,9 ml) was adjusted with 2 HCl to pH 4.4 and 2.9 mg of dithiotreitol dissolved in 75 µl 0.9% sodium chloride was added. The reduction was completed in 30 min. The reaction solution was added to a Sephadex G25 NAP 10 column (Pharmacia Biosystems AB) and eluted with 1.5 ml of 0.1 M phosphate buffer pH 7.5 with 0.9% NaCl and immediately used in the synthesis of product Q in example 11.

Example 11. **Preparation of SEA-monoclonal antibody conjugate Q with double spacer**

Dodeca(17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoylamino)Mab C242 (C) (4.2 mg, 27 nmoles in 1.0 ml of 0.1 M phosphate buffer pH 7.5 with 0.9% NaCl) was reacted during 43 h with [17-(3-mercaptopropionylamino)-3,6,9,12,15-pentaoxaheptadecanoylamino]-rSEA (P) (1.17 mg, 42 nmoles in 1 ml of the above buffer) in the dark in nitrogen atmosphere. Thereafter 1.14 µmole of mercaptoethanol was added. After another 1 h the reaction solution was fractionated on a Superdex 200 HR 16/65 column. The product was eluated with 2 mM phosphate buffer pH 7.5 with 0.9 % NaCl. Fractions with the desired product Q were pooled and analysed as described in example 4B. The conjugate was composed of 9% Mab C242 with two SEA, 25% with one SEA and 66% of unsubstituted Mab C242.

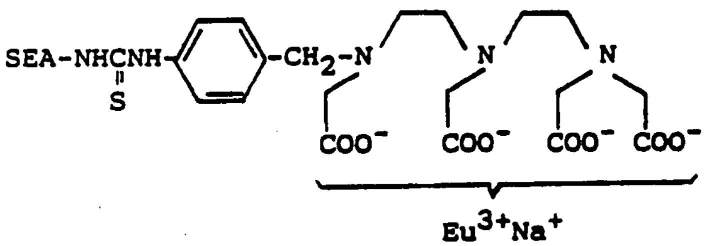
$ICH_2\underset{\underset{O}{\|}}{C}NHCH_2CH_2(OCH_2CH_2)_4OCH_2COOH$ A



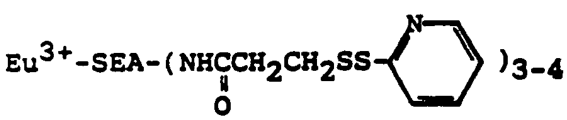
B

$IgG\text{-}(NH\underset{\underset{O}{\|}}{C}CH_2O(CH_2CH_2O)_4CH_2CH_2NH\underset{\underset{O}{\|}}{C}CH_2I)_n$ C

$n = 4, 7, 9, 12, 14, 18$



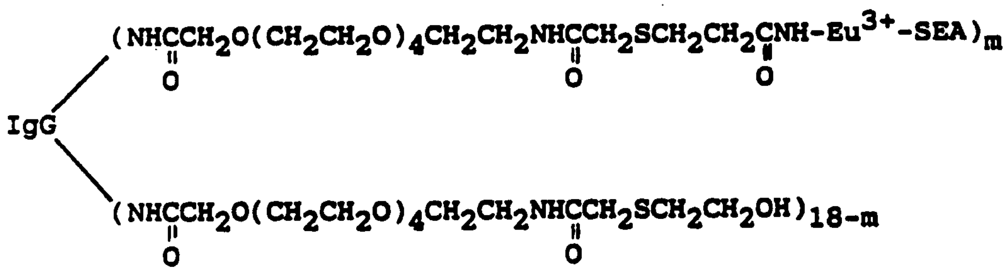
$(Eu^{3+}\text{-}SEA)$ D



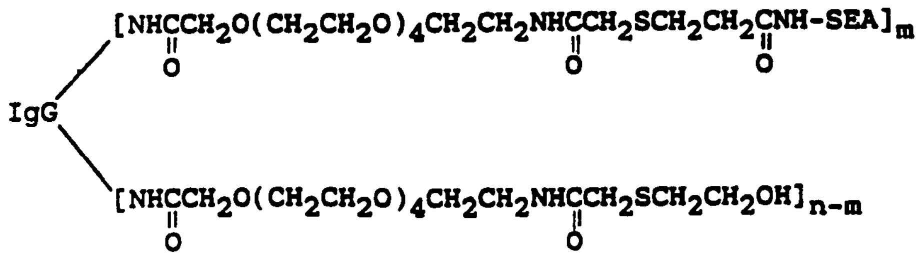
E

$Eu^{3+}\text{-}SEA\text{-}(NH\underset{\underset{O}{\|}}{C}CH_2CH_2SH)_{3-4}$ F1

$SEA(NH\underset{\underset{O}{\|}}{C}CH_2CH_2SH)_{1.9}$ F2

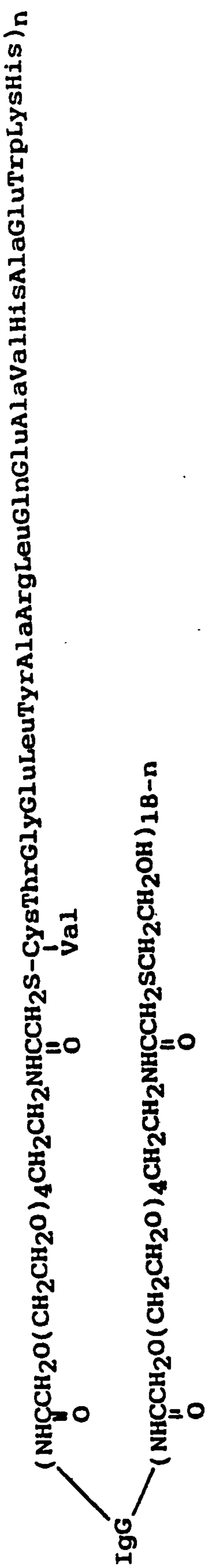


G1

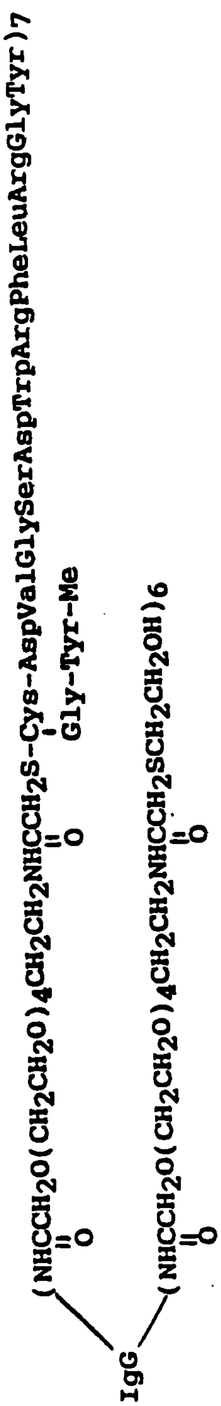


G2

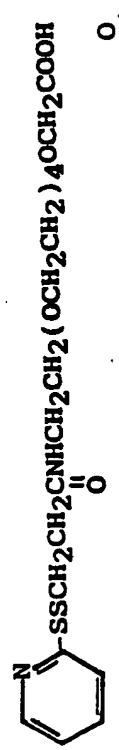
H



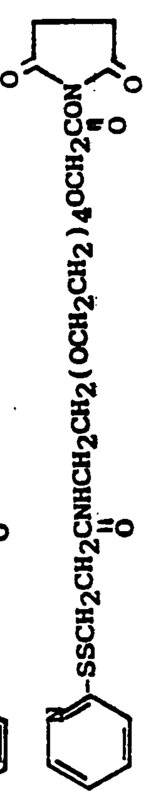
I



J

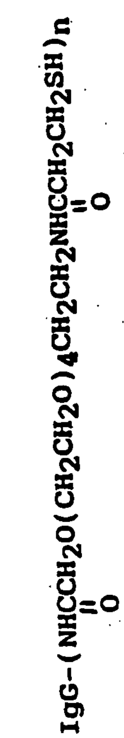


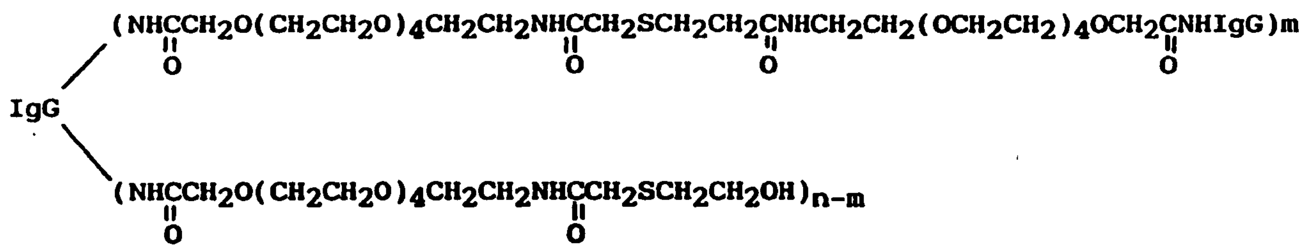
K



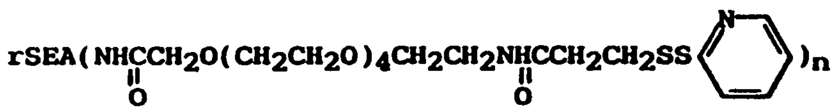
L

M





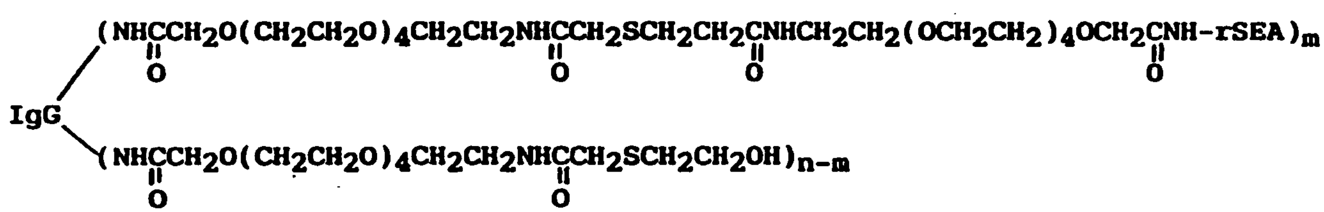
N



O

$$rSEA(NHC(=O)CH_2O(CH_2CH_2)_4CH_2CH_2NHC(=O)CH_2CH_2SH)_n$$

P



Q

EXPERIMENTAL PART II

Effects of superantigen-antibody conjugates on cells

The bacterial toxin used in the following experiments was Staphylococcus enterotoxin A (SEA) obtained from Toxin Technologies (WI; USA) or produced as a recombinant protein from E. Coli.

The antibodies were C215, C242 and Thy-1.2 mAbs. C215 is an lgG2a mAb raised against human colon carcinoma cell line and reacts with a 37kD protein antigen on several human colon cell lines. References to these mAbs have been given above. The conjugates were prepared as described in the preceding part.

Before the priority date studies had only been performed with Eu^{3+} labelled SEA-C215 mAb conjugates. During the priority year the results have been verified with unlabelled SEA-215, SEA-242 and SEA-Thy-1.2 mAb conjugates. The results now incorporated refer to unlabelled conjugates.

To determine the cytotoxicity mediated by the SEA-C215 mAb conjugate and unconjugated SEA and C215 mAb against colon carcinoma cells lacking MHC Class II or expressing low but undetectable amounts of MHC Class II, we employed various human SEA expanded T cell lines as effector cells and a panel of colon carcinoma cells and MHC Class II^+ Raji cells as target cells. The colon carcinoma cell lines Colo205, SW620 and WiDr, all lacked expression of MHC Class II, as determined by staining with mAbs against HLA-DR, HLA-DP and HLA-DQ and FACS analysis. The SEA expanded T cell lines were established from peripheral blood by weekly restimulations with mitomycin C treated MHC Class II^+ BSM lymphoma cells precoated with SEA in the presence of recombinant IL-2 (20 units/ml). These T cell lines were strongly cytotoxic towards Raji or BSM cells coated with SEA but not to uncoated cells or cells coated with staphylococcal nterotoxin B (SEB). This SEA induced killing is dependent on interaction of SEA with MHC Class II

on the target cell as determined by the use of blocking HLA-DR antibodies, MHC Class II$^-$ Raji mutant cells and HLA-DR transfected L-cells (Dohlsten et al., Immunology 71 (1990) 96-100. These T cell lines could be activated to kill C215$^+$ MHC Class II$^-$ colon carcinoma cells by the C215-SEA conjugate. In contrast unconjugated SEA and C215 mAb were unable to induce more than marginal T cell killing against the C215$^+$ MHC Class II$^-$ colon carcinoma cells. The staphylococcal enterotoxin antibody conjugate dependent cell-mediated cytotoxicity was dependent on binding of the SEA-C215 mAb conjugate to the C215$^+$ tumor cells. The specificity in this binding was demonstrated by the fact that excess of unconjugated C215 mAb but not the irrelevant C242 and w6/32 mAbs inhibited the lysis of the colon carcinoma cells. CD4$^+$ and CD8$^+$ T cells demonstrated killing of SEA-C215 treated C215$^+$ colon carcinoma cells, but did not lyse SEA treated cells. The interaction of T cells with SEA-C215 mAb conjugate bound to MHC Class II$^-$ tumor cell seems to involve interaction with specific V-beta TCR sequences in a similar manner as earlier demonstrated for SEA induced killing of MHC Class II$^+$ cells. This was indicated by the interaction of an SEA specific but not an autologous SEB specific T cell line with the C215-SEA conjugate. C242 mAb and Thy-1.2 mAb conjugates demonstrate activity in analogy with the C215 mAb conjugate.

Chromium labelling and incubation of the target cells with SEA

0.75×10^6 target cells and 150 µCi ^{51}chromium (Amersham Corp., Arlington Hights, England) were incubated for 45 minutes at 37°C in a volume of 100 µl. The cells were kept in complete medium containing RPMI-1640 medium (Gibco, Paisley, GBR) supplemented with 2.8 % (v/v) 7.5 % $NaHCO_3$, 1 % sodium pyrovate, 2 % 200 mM L-glutamine, 1 % 1M Hepes, 1 % 10 mg/ml gentamicin and 10 % fetal calf serum (FCS, Gibco, Paisley, GBR). After the incubation the cells were washed once in complete medium without FCS and incubated 60

minutes at 37°C and washed and resuspended in complete m dium containing 10 % FCS. $5x10^3$ target cells were added to each well of U-bottom 96-well microtiter plates (Costar, Cambridge, USA).

Cytotoxicity assay

The effector cells were added to the wells at various effector/target cell ratios. The final volume in each well was 200 µl. Each test was done in triplicate. The plates were incubated 4 hours at 37°C after which the released chromium was harvested. The amount ^{51}Cr was determined in a gamma-counter (Cobra Auto-gamma, Packard). The percentage cytotoxicity was computed by the formula % cytotoxicity = (X-M)/(T-M) * 100, where X is the chromium release as cpm obtained in the test sample, M is the spontaneous chromium release of target cells incubated with medium, and T is the total chromium release obtained by incubating the target cells with 1 % sodium dodecyl sulfate.

RESULTS

SEA-C242, SEA-C215 and SEA-anti-Thy-1.2 mAb conjugates bind to cells expressing the relevant epitopes of the mAbs, respectively, and to MHC Class II^+ cells. Unconjugated SEA on the other hand only binds to MHC Class II^+ cells. Unconjugated C215, C242 and Thy-1.2 mAbs bind to the relevant cells but not to Raji cells. (Table 1)

Human T cell lines lysed the MHC Class II^- SW620, Colo205 and WiDr cells in the presence of SEA-C215 mAb conjugate but not in the presence of unconjugated SEA and C215 mAb (Fig. 1). The lysis of colon carcinoma cells was seen at 10-100 ng/ml of SEA-C215 mAb conjugate. High levels of lysis at various effector to target ratios were seen with SEA-215 mAb conjugate against SW620 (Fig. 1). In contrast, unconjugat d SEA or C215 mAb mediated no cytotoxicity against SW620 cells at all tested effector to target ratios.

This indicates that the capacity to lyse MHC Class II^- Colo205 cells is restricted to the conjugat and cannot be induced by unconjugated SEA and C215 mAb. SEA and SEA-C215 mAb conjugate but not C215 mAb mediated T cell killing of MHC Class II^+ Raji cells and of interferon treated MHC Class II^+ Colo205 cells (Fig. 1).

In order to demonstrate that the SEA-C215 mAb conjugate mediated lysis involved specific binding of the conjugate to the C215 mAb molecule on the target cells, we performed blocking studies with excess of unconjugated C215 mAb and mAb C242, which bind to an irrelevant antigen on the colon carcinoma cells (in regard to C215 mAb binding). Addition of mAb C215 strongly blocked cytotoxicity, whereas the C242 mAb had no influence (Fig. 2). Similarly lysis by a SEA-C242 mAb conjugate was specifically blocked by excess of unconjugated C242 mAb but not C215 mAb.

The capacity of SEA-C215 mAb conjugate to induce T cell dependent lysis of MHC Class II SW620 colon carcinoma cells was seen in both $CD4^+$ and $CD8^+$ T cell populations (Table 2). SEA did not activate any of these T cell subsets to mediate killing of SW620 cells but induced lysis of MHC Class II^+ Raji cells (Table 2).

The SEA-C215 mAb conjugate induced lysis of SW620 and Raji cells by a SEA expanded T cell line, but not by a SEB expanded T cell line (Fig. 3). The specificity of the SEA and SEB lines is indicated by their selective response to SEA and SEB, respectively, when exposed to Raji cells (Fig. 4). This indicates that the SEA-C215 mAb conjugate retains similar V-beta TCR specificity as for unconjugated SEA.

Legend to figures

Fig. 1. The SEA-C215 mAb conjugate directs CTLs against MHC class II^- colon carcinoma cells. Upper left panel demonstrates the effect of SEA responsive CTLs against SW620 cells at various effector to target ratios in the absence (-) or pr senc of SEA-C215 mAb conjugate, SEA, C215 and a

mixture of C215 and SEA (C215+SEA) at a concentration of 1 μg/ml of each additive. The other panels demonstrates the capacity of SEA-C215 mAb conjugate, and SEA to target SEA responsive CTLs against the C215+MHC class II^- colon carcinoma cell lines SW620, Colo205 and WiDr, MHC class II^+ $C215^+$ interferon treated Colo205 cells and C215- MHC class II^+ Raji cells. Effector to target ratio was 30:1. Addition of unconjugated C215 mAb, at several concentrations, did not induce any CTL targeting against these cell lines. FACS analysis on SW620 cells, Colo205 and WiDr cells using mAbs against HLA-DR, -DP, -DQ failed to detect any surface MHC class II expression, whereas abundant expression of HLA-DR, -DP and -DQ was detected on Raji cells and HLA-DR and -DP on interferon treated Colo205 cells. Colo205 cells were treated with 1000 units/ml of recombinant interferon-gamma for 48 hours prior to use in the CTL assay.

Fig. 2. SEA-C215 mAb conjugate and SEA-C242 mAb conjugate induced CTL targeting against colon carcinoma cells depends on the antigen selectivity of the mAb. Lysis of Colo205 cells by a SEA responsive CTL line in the presence of SEA-C215 mAb and SEA-C242 mAb conjugate (3 μg/ml) is blocked by addition of unconjugated C215 and C242 mAbs (30 μg/ml), respectively. The unconjugated mAbs or control medium (-) were added to the target cells 10 minutes prior to the conjugates.

Fig. 3. Lysis of SEA-C215 mAb conjugate coated colon carcinoma cells is mediated by SEA but not SEB responding CTLs. Autologous SEA and SEB selective T cell lines were used at an effector to target ratio of 10:1 against SW620 and Raji target cells in the absence (control) or presence of SEA-C215 mAb conjugate, a mixture of unconjugated C215 mAb and SEA (C215+SEA) and unconjugated C215 mAb and SEB (C215+SEB) at a concentration of 1 μg/ml of each additive.

Fig. 4. Cytotoxicity induced by the SEA-C242 mAb conjugate and SEA-Anti-Thy-1.2 mAb conjugate against th ir target cells (Colo205 tumour cells and EL-4 tumour cells, respectively).

Table 1

SEA-C215 mAb conjugate bind to C215^{+} colon carcinoma cells and MHC Class II^{+} Raji cells

Reagent	Cell	Facs analysis
SEA-C215 mAb	Colo205	Pos
	Raji	Pos
C215 mAb	Colo205	Pos
	Raji	Neg
SEA-C242 mAb	Colo205	Pos
	Raji	Pos
C242 mAb	Colo205	Pos
	Raji	Neg
SEA-anti-Thy-1.2 mAb	EL-4	Pos
anti-Thy-1.2 mAb	EL-4	Pos
SEA	Colo205	Neg
	Raji	Pos
control	Colo205	Neg
	Raji	Neg
	EL-4	Neg

Cells were incubated with the various additives of control (PBS-BSA) for 30 minutes on ice, washed and processed as described below. The staining of C215 mAb and C242 mAb bound to Colo205 cells and anti-Thy-1.2 bound to EL-4 cells was detected using FITC labelled rabbit anti mouse 1 g. The staining of SEA to Raji cells was detected using a rabbit anti-SEA sera followed by a FITC-swine anti rabbit 1 g. The staining of SEA-C215 mAb conjugate to Colo 205 and Raji cells was detected utilizing the above described procedures for C215 mAb and SEA. FACS analysis was performed on a FACS star plus from Becton and Dickinson. Staining with second and third steps only was utiliz d to define the background.

Table 2

$CD4^+$ and $CD8^+$ CTLs lyse colon carcinoma cells presenting the C215-SEA conjugate.

		% cytotoxicity		
Effector[A)]	Target	control	SEA	C215-SEA
$CD4^+$	SW620	2	5	50
$CD4^+$	Raji	0	41	43
$CD8^+$	SW620	0	1	23
$CD8^+$	Raji	2	72	68

A) The CTLs (SEA-3) were used at effector to target ratios of 30:1 in the absence (control) or presence of SEA and C215-SEA at 1 µg/ml.

PATENT CLAIMS

1. A conjugate substance (A-B-A') where A and A' comprise residues from organic compounds F and F', respectively; at least one of which being a polymer (carrier) and said compounds having properties that are retained in the conjugate, and -B- being a bridge that is covalently bound to A and A',
characterized in that the bridge -B- comprises the structure

$$-S_rRCONHCH_2CH_2(OCH_2CH_2)_nO(CH_2)_mCOY- \qquad (I)$$

where

(i) n is an integer, for instance 1-20 and preferably > 2, and such that n is uniform for bridges linking identically located positions in individual molecules of the conjugate (conjugate substance);

(ii) m is 1 or 2;

(iii) R is an alkylene group of 1-4 carbon atoms, preferably < 2 carbon atoms, possibly substituted with one or more (1-3, preferably < 2) hydroxy(OH) groups; (iv) S_r binds to saturated carbon atoms in both directions, and r = 1 or 2;

(v) Y is -NH-, -NHNH- or -NHN=CH- that in their left ends bind to CO and in their right ends to saturated carbon atom or to a carbonyl group (only -NHNH-).

2. A conjugate according to claim 1, **characterized** in that the polymer is a biopolymer exhibiting a polypeptide and/or polysaccharide structure.

3. A conjugate according to any of claims 1-2, **characterized** in that the polymer is an antibody or an antibody active fragment thereof.

4. A conjugate according to any claims 1-3, **charact rized** in that the polymer is soluble in aqueous media.

5. A conjugate according to claim 3, **characterized** in that the entity of A and A′ that is not the antibody or the antibody active fragment is an analytically detectable group.

6. A conjugate according to any of claims 1-5, **characterized** in that the carrier polymer is an antibody or an antibody active fragment thereof, and that the entity of A and A′ that is not the carrier polymer is an immune stimulator, e.g. of bacterial origin.

7. A bifunctional coupling reagent complying with the formula

$$Z_1RCONHCH_2CH_2(OCH_2CH_2)_nO(CH_2)_mZ_1' \qquad (III)$$

where
(i) n is an integer, for instance 1-20 and preferably > 2,
(ii) m is an integer 1 or 2, preferably 1,
(iii) Z_1 is a SH-reactive electrophile or thiol (SH-) or protected thiol, for instance acylated thiol such as AcS-,
(iv) R is alkylene (1-4 carbon atoms, preferably < 2 carbon atoms) that possibly is substituted with one or more (1-3, in the preferred case < 2) hydroxy (OH) groups.
(v) Z_1' is activated carboxy.

8. A bifunctional coupling reagent according to claim 7, **characterized** in that Z_1 is selected among reactive disulfide in which one of the sulfur atoms binds to R; mercapto; 2,5-dioxo-1-aza-cyclopent-3-en-1-yl; and halo, preferably bromo or iodo.

9. A polyether, **characterized** in that it complies with the formula:

$$XCH_2CH_2(OCH_2CH_2\text{-})_nOCH_2Y \qquad (IV)$$

where
n is an integer 2-20, preferably 3-10;

X is is H_2N- or substituted H_2N- that is transformable to H_2N-, preferably by hydrolysis or reduction, for instance (a) nitro; (b) amido (=carbamido), such as lower saturated acylamido; phtalimidoyl; carbamato; lower alkylamino in which the substituting carbon atom is alpha to an aromatic system; and (d) 4-oxo-1,3,5-triazin-1-yl;

Y is carboxy (-COOH or $-COO^-$) or a group that is transformable to carboxy, preferably by hydrolysis or oxidation, for instance
(a) an ester group in which the carbonyl carbon atom or a corresponding atom binds to the methylene in the right terminal of formula (I), or
(b) -CHO, (c) -CN, $-CONH_2$, $-CONR_1'R_2'$, where R_1' and R_2' are lower alkyl, particularly secondary and tertiary alkyl groups, methyl that is substituted with 1-3 phenyl groups that possibly are ring substituted.

10. A polyether according to claim 9, **characterized** in that X is NH_2-.

11. A polyether according to claim 9, **characterized** in that X is acylamido, such as CH_3CONH- and CH_3CONH- having an electron-withdrawing substituent on the alpha carbon atom (e.g. CF_3CONH- or CH_3COCH_2CONH-), and formylamido (HCONH-).

12. A polyether according to claim 9, **characterized** in that X is phtalimidoyl or carbamato, such as $R_1'OCONH$- and $(R_1'OCO)(R_2'OCO)N$-, where R_1' and R_2' may be a lower alkyl group, particularly secondary and tertiary lower alkyl groups, or a methyl group that is substituted with 1-3 phenyl groups that possibly are ring substituted.

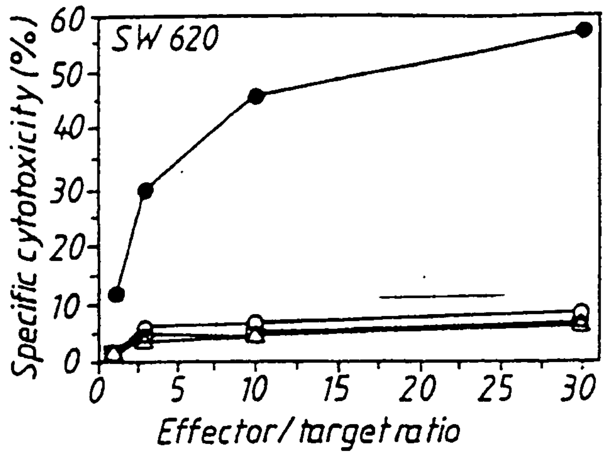
13. A polyether according to any of claims 9 or 12, **characteriz d** in that X is Boc, Z or diZ.

14. A polyether according to claim 9, **characterized** in that X is an alkylamino group, where the carbon atom substituting on the nitrogen atom is alpha to an aromatic system, such as N-monobenzylamino.

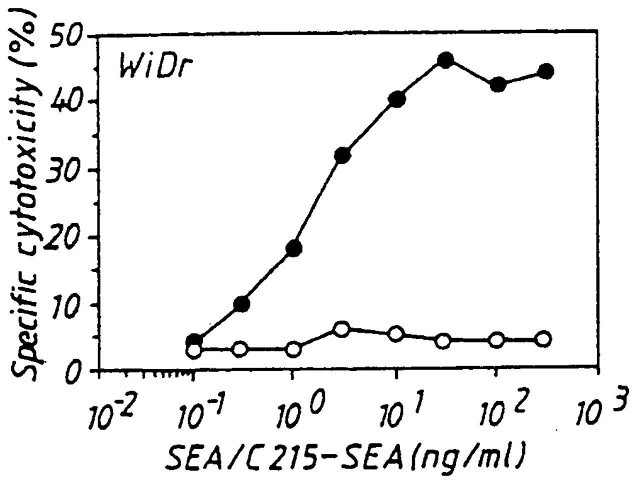
15. A polyether according to any of claims 9-14, **characterized** in that Y is a carboxy group.

16. A polyether according to claim 9, **characterized** in that Y is an ester group binding at its carbonyl carbon atom or at a corresponding atom to the methylene in the right terminal of formula (IV).

17. A polyether according to claims 9 or 16, **characterized** in that Y is an alkyl ester group ($-COOR_1'$); an ortoester group ($-C(OR_2')_3$) or a reactive ester group, such as N-succinimid-1-yloxycarbonyl, 4-nitrophenyloxy-carbonyl and 2,4-dinitrophenyloxycarbonyl, R_1' and R_2' being a lower alkyl group, particularly a secondary or a tertiary alkyl group, and a methyl group that is substituted with 1-3 phenyl groups that possibly are ring substituted.



F I G. 1a



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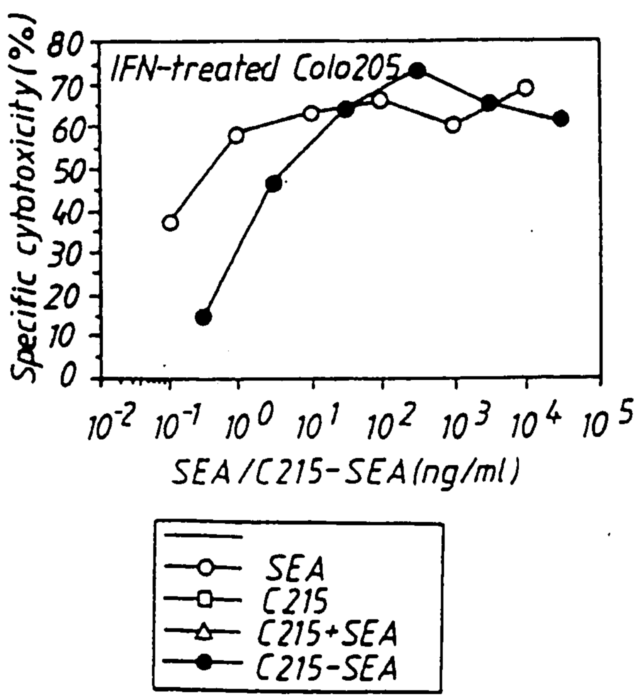
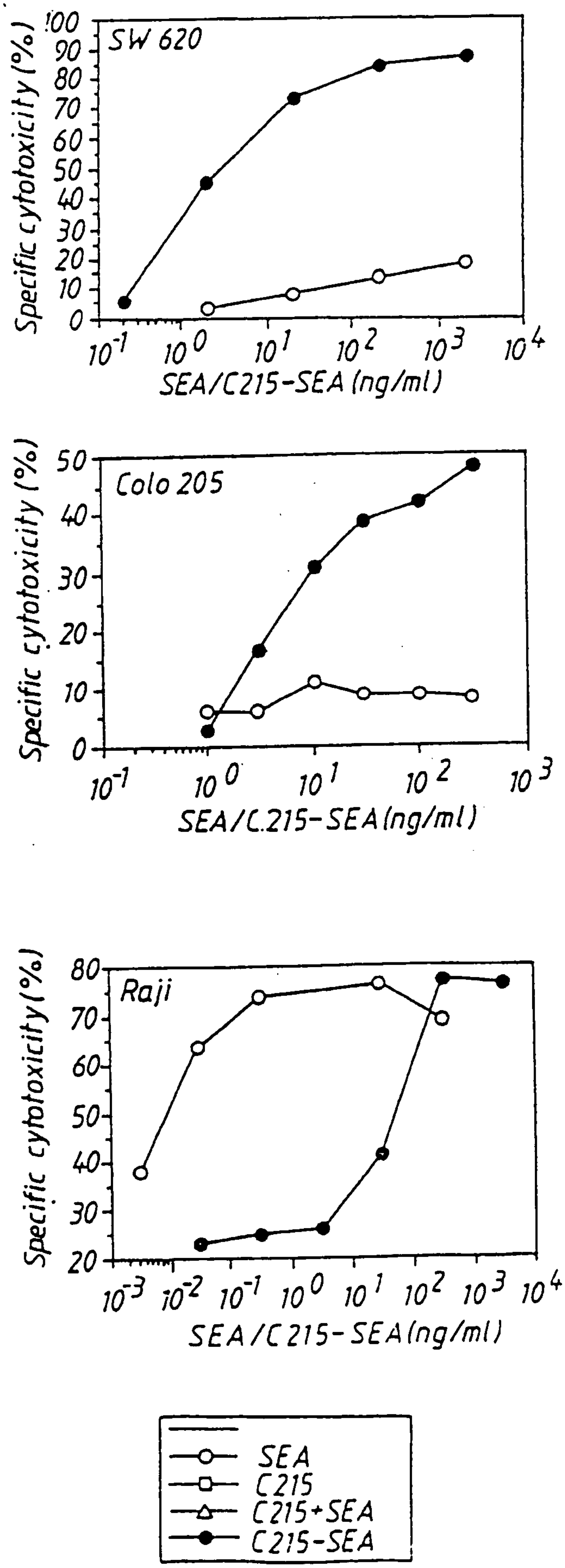


F I G. 1c

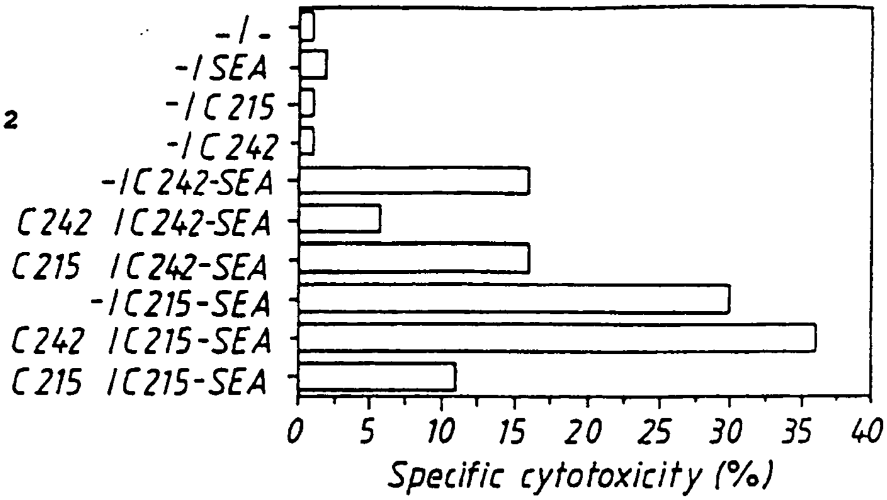
F I G. 1d

F I G. 1e

F I G. 1f



F I G. 2



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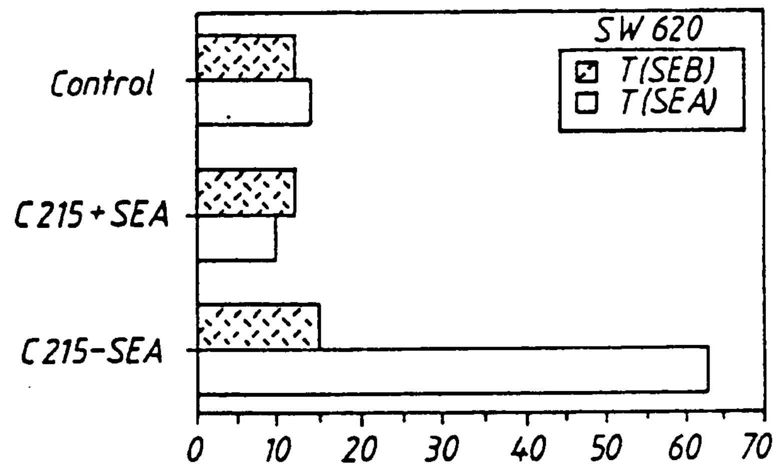
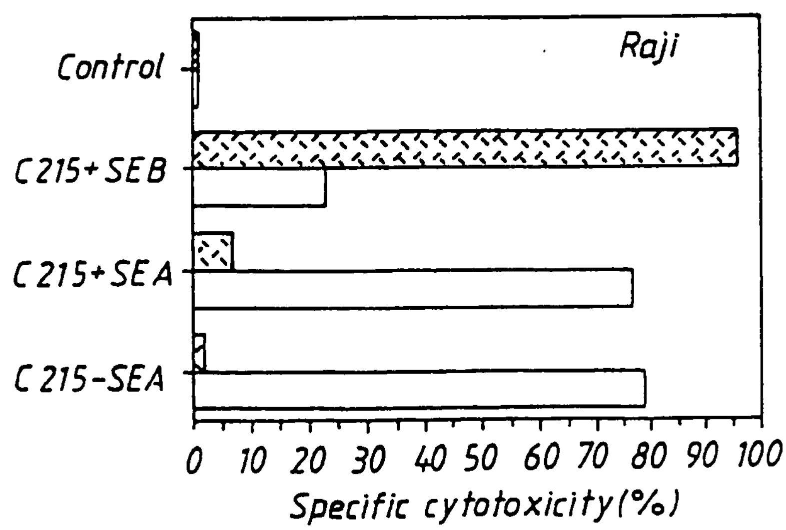
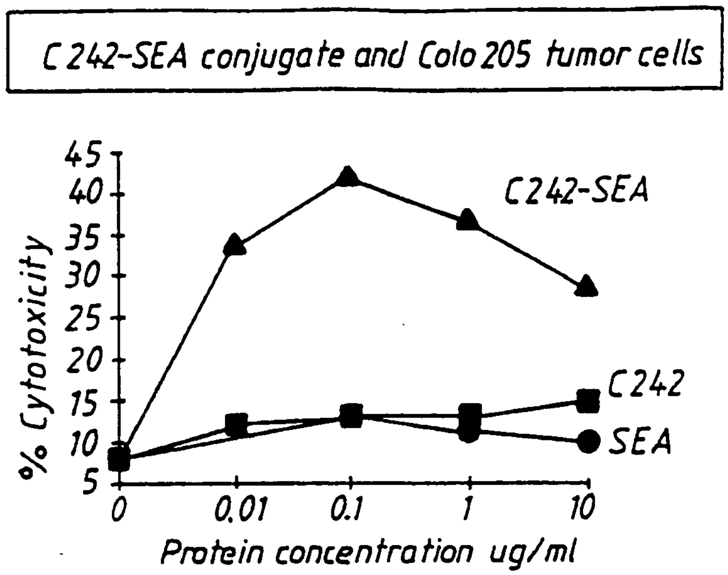


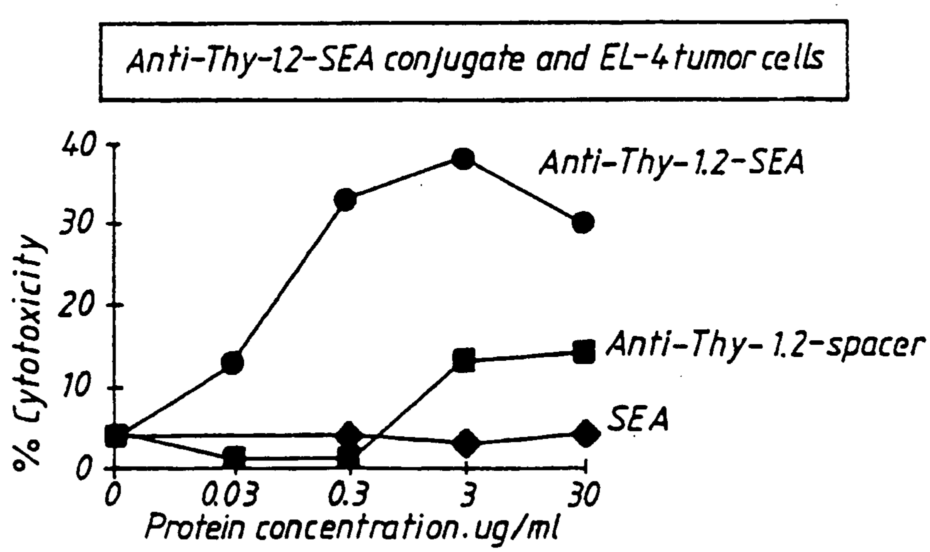
F I G. 3b



F I G. 4a


C 242-SEA conjugate and Colo 205 tumor cells
% Cytotoxicity
45
40
35
30
25
20
15
10
5
C242-SEA
C242
SEA
0
0.01
0.1
1
10
Protein concentration ug/ml

F I G. 4b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% Cytotoxicity
40
30
20
10
0
Anti-Thy-1.2-SEA
Anti-Thy- 1.2-spacer
SEA
0
0.03
0.3
3
30
Protein concentration.ug/ml

NOTIFICATION CONCERNING THE RESULT OF THE PARTIAL INTERNATIONAL SEARCH

International Application No PCT/SE 91/00497

This notification is an annex to the invitation to pay additional fees issued in accordance with Article 17 (3) (a) and Rule 40.1 PCT.
It gives, for information, the result of the international search carried out on those parts of the international application which relate to the invention first mentioned in the claims.

This notification must not be confused with the international search report provided for in Article 18 and Rule 43 PCT which will be drawn up in due course.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: A 61 K 39/44, C 07 D 209/48, C 07 C 217/09//A 61 K 39/385
C 07 K 3/08, G 01 N 33/53, C 07 K 17/06

II FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
IPC 5	C 07 D; C 07 C; G 01 N; C 07 K; A 61 K

III DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	EP, A1, 0410280 (BOEHRINGER MANNHEIM GMBH) 30 January 1991, see the whole document --	1-7
X	Tetrahedron Letters, Vol. 29, No. 31, 1988 L Jullien et al.: "The"CHUNDLE" Approach to Molecular Channels Synthesis of a Macrocycle-based Molecular Bundle", see page 3803 - page 3806 see molecules 3 and 5 on page 3804 --	7
X,Y	EP, A2, 0240200 (CETUS CORPORATION) 7 October 1987, see page 3, line 50 - line 52; page 4, line 5 - line 36; page 6, line 20 - line 35; page 9	1-6
Y	--	7

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

22nd October 1991

1991 -10- 25

International Searching Authority

SWEDISH PATENT OFFICE

Signature of Authorized Officer

Carl-Olof Gustafsson

Form PCT/ISA/206 (Annex)(January 1989)

International Application No. PCT/SE 91/00497

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FR M THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X,Y Y	WO, A2, 8912624 (CETUS CORPORATION) 28 December 1989, see pages 9-16 and definition of R2 on pages 10-12, pages 22-23 --	1-6 7
A Y	EP, A2, 0345789 (TAYLOR, KEITH E.) 13 December 1989, see the whole document --	1-6 7
A X	WO, A1, 8803412 (CETUS CORPORATION) 19 May 1988, see page 15, line 11 - line 15; page 16 - page 17 --	1-6 7
A	Chemical Abstracts, volume 111, no. 12, 18 September 1989, (Columbus, Ohio, US), Nagae, S et al.: "Development of coupling agent with long-chain hydrophilic spacer. Application to protein modification ", see page 351, abstract 102661u, & Jinko. Zoki 1989, 18(1), 137- 140 -- --------	1

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers..........., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers..........., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers..........., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional saech fees.

The acceptance of a single general inventive concept covering end products as well as products used to prepare these end products (intermediates) implies that when several claimed intermediates are implied in different reactions, these intermediates are technically closely inter-connected with the end products as well as with themselves by their use for incorporation of the same essential structural part into the end products.

This is not the case for the intermediates stipulated in the claims 9-17

Therefore, a single general inventive concept based on the relationship intermediates/end products is lacking and this leads to the subjects as listed below, each falling under its own restricted inventive concept, defined by the nature of the essential structural part present in each intermediate and incorporated into the end product(s).

1. Claims 1-8: conjugates and bifunctional couplong reagents (intermediates)

2. Claims 9-17: polyethers (intermediates in the production of the bifunctional coupling reagents).

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 91/00497

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-09-27
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A1- 0410280	91-01-30	DE-A-C-	3924705	91-01-31
		DE-A-	3943522	91-02-07
		JP-A-	3068542	91-03-25
EP-A2- 0240200	87-10-07	JP-A-	62252759	87-11-04
		US-A-	4797491	89-01-10
		US-A-	5034514	91-07-23
WO-A2- 8912624	89-12-28	AU-D-	3839089	90-01-12
		EP-A-	0428534	91-05-29
EP-A2- 0345789	89-12-13	NONE		
WO-A1- 8803412	88-05-19	AU-D-	8326487	88-06-01
		EP-A-	0305409	89-03-08
		US-A-	4894226	90-01-16